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Aerobic exercise training is a beneficial stress that causes physiological changes to adapt to many different forms of stress. Oxidative stress is a form of stress that is classified by the production and imbalance of harmful radicals, termed reactive oxygen and nitrogen species (RONS). Cells within the body can produce antioxidants to act as a defense against these dangerous molecules and potential future RONS. An imbalance in antioxidant production to oxidative stress can lead to cell dysfunction or cell death, which may lead to diseased states. A normal response to an increase in RONS leads to activation of a transcription factor, nuclear erythroid 2-related factor 2 (Nrf2), which signals the production of antioxidants. As such, Nrf2 has emerged as the 'master regulator' of antioxidant production. However, Kelch-like ECH Associated Protein 1 (KEAP1) is the protein that is responsible for Nrf2 regulation and activation. While this pathway has been implicated as a major role in redox homeostasis, much remains unknown about its response to exercise. Therefore, the purpose of this study was to evaluate the changes that occur in the KEAP1/Nrf2/Antioxidant pathway to exercise training.

In order to assess these changes, mice (C57BL/6) were assigned to two groups: 1) underwent an 8-week exercise training program (n = 14) and 2) were sedentary control (n = 9). The exercise training program consisted of treadmill running 5 days per week initially for 45 minutes to 1 hour and progressed each week by adding duration, speed, and incline, ending at approximately 65% VO_2max intensity. Seventy-two hours after the

last session, mice were sacrificed; quadriceps, gastrocnemius, and soleus hindlimb muscles were harvested and stored for biochemical analysis. Multiplexed fluorescent western blot analysis was used to quantify KEAP1, nuclear Nrf2, cytosolic Nrf2, SOD1, and HO1 protein levels; the latter two proteins are related to controlling RONS. Because multivariate assumptions were violated and power was low, univariate repeated-measures ANOVAs were used to assess the differences between sedentary and exercise groups, along with potential differences between muscles.

Simple main effects illustrated significant differences between exercise groups in the gastrocnemius ($F = 4.438$, $p = 0.047$), soleus ($F = 6.082$, $p = 0.022$), and deep quadriceps ($F = 10.756$, $p = 0.004$). SOD1, KEAP1, and cytosolic Nrf2 fraction all approached significance for a main effect of exercise between groups ($p = 0.062$, $p = 0.111$, $p = 0.104$, respectively). Additionally, HO1 and SOD1 each demonstrated a significant effect of muscle ($p < 0.001$, each). The Nrf2/KEAP1 (both cytosolic and total ratios) did not differ between muscle groups or between exercise groups and did not significantly predict antioxidant level production. It appears that exercise training, according to this protocol, increases nuclear translocation in a muscle-dependent fashion. Future studies should address complications with the current study, particularly low power from low sample size, as well as genetic regulations explaining control between Nrf2 activation and down-stream antioxidant production.

THE ROLE OF KEAP1 IN NRF2 REGULATION IN RESPONSE TO CHRONIC EXERCISE TRAINING

by

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CHAPTER I

INTRODUCTION

Cells in the body may produce reactive oxygen nitrogen species (RONS), that have an oxygen or nitrogen group that could interact with and damage molecules in close proximity to the RONS molecules[1]. RONS may lead to cellular damage of structural proteins and enzymes, which could alter the way a cell functions; additionally, RONS molecules may act as secondary messengers, triggering the activation of intracellular pathways that protect the cells[2]. These pathways, outlined later, are vital in a cell's overall health and function. The responses to the RONS can trigger adaptations in a cell, and if the adaptations are insufficient, then some cells may become dysfunctional and lead to diseased states[3].

Generally, RONS are produced with increased metabolism or during times of infection/ inflammation[4]. When the production of these RONS increase above normal basal levels, this situation has been denoted as oxidative stress (OS). It is typically thought that OS, like many other stresses, is regulated in a manner called 'hormesis'. Hormesis means there is a certain increase in stress that may lead to beneficial adaptations to the cells to protect against future stress; however, if stress becomes too high to manage, a cell may undergo apoptosis (cell death); lastly, if there is too little stress the system is not regulated with the proper protections. Thus, it is important to have some OS to protect future insults, but too much may cause irreversible damage.

Exercise, inflammation, and infection are common causes of OS. Infection and inflammation lead to the production of RONS in order to cause damage to pathogens or cells that are damaged[5]. This mechanism helps to 'kill' invaders and infected cells. Alternatively, exercise leads to an increase in RONS, thought to be a byproduct of increased aerobic metabolism and alteration of shear stress within the circulation, among other processes to regulate proper functions within the body[6]. Some processes still need to be elucidated. Despite not fully understanding the mechanisms which produce RONS production, exercise has been demonstrated to transiently increase RONS, intracellular damage, and subsequent antioxidant production[7–10].

There are many antioxidants that exist in most cell types in the human body, and many of them respond to single or repeated bouts of OS, as in chronic exercise training, by increasing total quantities of the antioxidant[11]. Many of these antioxidants are produced through NRF2 signaling, which is a transcription factor and is currently considered a 'master regulator' of antioxidant production in the cell. Upon activation, NRF2 located within the cytosol is translocated to the nucleus and binds to genes that contain an "antioxidant response element" (ARE). The activation of NRF2 is controlled by a process to release the protein KEAP1 from its attachment to NRF2 within the cytosol, which responds to acute OS, freeing NRF2, and allowing it to translocate into the nucleus and promote transcription of ARE-genes[12]. This is shown in figure 1.

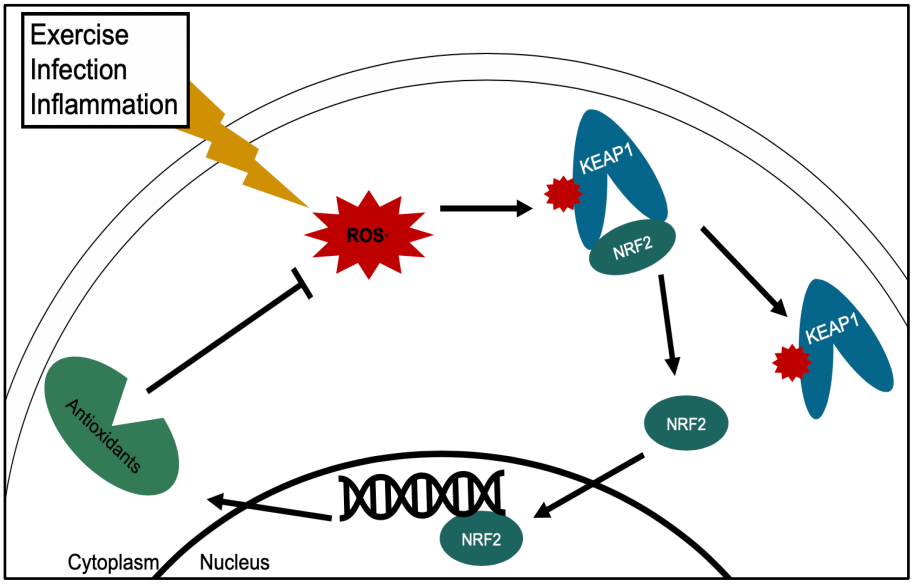


Figure 1. Conceptual model of KEAP1/Nrf2 activation in response to stressors.

Research has focused on changes in NRF2 concentration in response to acute and chronic exercise training, but many of the results to date show conflicting results in the relationship between NRF2 levels and down-stream antioxidant production[13]. While there are many factors that may affect the relationship between a signal and production of a protein, one major factor that may be overlooked in these studies is the KEAP1 molecule. KEAP1 is a protein that sequesters NRF2 in the cytosol of the cell, rendering it inactive or controlling its degradation through ubiquitin ligases. Upon OS, KEAP1 may release NRF2, allowing it to become active. Thus, the amount of KEAP1 may be important to consider in relationship to the amount of NRF2. Changes observed in NRF2 relative to changes in KEAP1 may help better explain the bioavailability of NRF2 and suggest the NRF2 activity changes.

Therefore, the purpose of this study is to examine the changes in KEAP1 and Nrf2 in response to chronic exercise training, and to examine the importance of the KEAP1/NRF2 ratio in the cytosol to help explain the change in antioxidant levels.

Specific Aims

Specific Aim 1: To determine the redox adaptations that occurs in hindlimb skeletal muscle due to aerobic exercise training.

Hypothesis 1: SOD1 concentration will be higher in endurance trained muscle compared to sedentary muscle.

Hypothesis 2: HO1 concentration will be higher in endurance trained muscle compared to sedentary muscle.

Hypothesis 3: TGSH concentration will be higher in endurance trained muscle than sedentary muscle.

Specific Aim 2: To determine changes in NRF2 concentration in muscle

Hypothesis 1: Total Nrf2 will be greater in trained muscle compared to Total Nrf2 in sedentary muscle

Hypothesis 1: Cytosolic Nrf2 will be greater in trained muscle compared to Cytosolic Nrf2 in sedentary muscle

Hypothesis 2: Nuclear Nrf2 will be greater in trained muscle compared Nuclear Nrf2 in sedentary muscle.

Specific Aim 3: To assess the changes of KEAP1 and its ratio with NRF2 in response to exercise training.

Hypothesis 1: KEAP1 concentration will be higher in endurance trained muscle compared to sedentary muscle.

Hypothesis 2: Nrf2/KEAP1 ratio will be higher in trained muscle than the sedentary Nrf2/KEAP1 ratio.

Specific Aim 4: Evaluate the effectiveness of the Nrf2/KEAP1 ratio in predicting antioxidant levels due to training.

Hypothesis 1: There will be a significant relationship between the amount of Nrf2/KEAP1 and the amount of antioxidant levels within the muscles examined.

Hypothesis 2: There will be a significant relationship between the change in the amount of Nrf2/KEAP1 and the change in amount of antioxidant levels after exercise training.

Study Significance

Many diseases have been implicated in having antioxidant deficiencies, such as neurodegenerative diseases, cardiovascular diseases, diabetes, among others. In many cases, exercise has been shown to attenuate disease progression, but through unknown mechanisms. It stands to reason that upregulation of protective antioxidants may attenuate oxidative stress, prevent damage, and promote cell survival in such diseases. Indeed, some studies demonstrate increases in antioxidant production that is concurrent with disease attenuation[14,15], suggesting that these antioxidants may play a protective role. However, the mechanisms that promote the enhancement of antioxidants have not fully been elucidated.

Exercise is a therapy that is used for many diseases and may be prescribed as a therapeutic means. As such, prescriptions often include intensity, duration, and frequency of the exercise, as these factors have all been shown to stimulate different adaptations. The specificity of these variables determines the efficacy of exercise training to ameliorate certain diseases. Thus, a good understanding of how each of these variables may change the production of antioxidants is required to evaluate the levels of intensity, duration, and frequency of exercise are the best for each disease. Since the response of KEAP1 and NRF2 to exercise are relatively unknown, efficacy may not yet be best determined. Having a better understanding of how KEAP1 and NRF2 change with exercise will allow for a better understanding of the relationship between these two factors and how ARE-related antioxidants are activated by endurance exercise.

Study Innovation

While there are many studies on the oxidative stress that occurs during exercise, the effects of exercise training on the pathways that control redox hormesis remain unknown. To our knowledge, this is the first study that attempted to examine the effects of exercise training on KEAP1 changes in skeletal muscles. A few studies have assessed acute and chronic changes to Nrf2 activation, but only two studies have included KEAP1 changes along with these measures, neither of which are in skeletal muscle. This study is innovative in that it provides a better understanding of the robust changes in oxidative stress signaling that occurs with endurance aerobic exercise training.

Additionally, as this study is an exercise training study, it provides more information into the protective effects of exercise against oxidative stress signaling

diseases. In many cases, such as cancer[16], cardiovascular disease[17], neurodegenerative diseases[18], and diabetes[19], there are abnormalities in the redox status and capacity to respond to these abnormalities. With a better understanding of KEAP1's role over Nrf2, this study provides more insight into how exercise may play a protective role in the pathology of these diseases.

Limitations

- Nuclear Nrf2 is not indicative of activity per se, but rather release and translocation of Nrf2 from KEAP1
- Nrf2 can be degraded by KEAP1, and rate of degradation will not be examined in this study.
- Mice were the model organism and thus the results may not be directly translatable to humans.
- Muscles were harvested at rest (basal) 48 hours after the last training exercise session, and therefore do not reflect a difference in response to an acute exercise session.

Delimitations

- Sedentary mice underwent the same timeline as the exercise intervention group; the sedentary group remained sedentary until the same age as the exercise mice at sacrifice.
- All mice were from the same genetic line.
- All mice had free access to food and drink in their cages.
- All mice were housed in the same environmental conditions.

- All mice were familiarized with the environment for at least 10 days prior to any interventions.

Term Definitions

GSH - Glutathione in its reduced form; representative of eustress conditions.

GSSG - Glutathione in its oxidized form; representative of increased oxidative stress.

HO - Heme oxygenase – an enzyme that is responsible for making bilirubin (an antioxidant) from heme degradation.

HPLC - High performance liquid chromatography, a method used to isolate molecules in a solution using a high pressure solution passing through a column to adhere and elute molecules for detection, often with electrochemical or mass-spectroscopy detection.

KEAP1- Kelch-like ECH Associated Protein 1, a protein that is responsible for sequestering Nrf2 in the cytosol. Also associated with Cullin-3 E3 ubiquitin ligase, which is an ubiquitinator of Nrf2 for 26S proteasome degradation.

Nrf2 - Nuclear erythroid 2-related factor 2 – a transcription factor that is considered to be a 'master regulator' of antioxidant and detoxification genes. Normally located in the cytosol bound to Keap-1.

OS - Oxidative stress – a scenario when the redox status of a cell favors oxidation due to an increase in RONS.

p62 - also known as Sequestosome 1 or SQSTSM1 – a protein that is associated with autophagy, adhering polyubiquinated proteins for selective autophagy.

RONS - Reactive oxygen and nitrogen species – molecules that contain radicalized oxygen or nitrogen groups, making them highly reactive and potentially damaging to other molecules around them.

SOD - Superoxide Dismutase – An enzyme that is responsible for the conversion of a superoxide molecule to a hydrogen peroxide molecule.

TGSH - Total Glutathione – The total amount of glutathione, both oxidized and reduced in a cell; often used to normalize GSSG data.

CHAPTER II

REVIEW OF LITERATURE

Reactive Oxygen Species

Oxygen is essential for proper cellular function, providing an electron acceptor for ATP production. By itself, oxygen can be a very stable molecule. However, due to the nature of its electrons, oxygen has the potential to become a highly reactive molecule. It is common for oxygen to become radicalized, which refers to an oxygen atom containing an unpaired electron in its valence shell. This molecule is very reactive, as electrons have a preference to be shared. In this case, the unpaired electron will try to pair with atoms around it, especially from other molecules. In the cell, these radicalized oxygen molecules can take electrons from molecules around it, referred to as 'oxidizing' the molecule, or 'reducing' the radical. When cellular components become oxidized, it may change the nature of how that molecule can function. Therefore, if many of the molecules in the cell become oxidized and cannot function properly, a cell may become dysfunctional or even lead to programmed cell death (apoptosis). This damage that comes from radicals has been suggested to lead to many chronic diseases, such as neurodegenerative diseases[18,20,21], cardiovascular disease[22,23], diabetes[19,24], and progression of cancer[16,25–27].

There are many kinds of radicals that can occur within a typical biological context. Molecules that contain oxygen or nitrogen atoms that become reactive and lead to oxidative stress are often called reactive oxygen or nitrogen species (RONS). Superoxides are one of the most common types of radicals in living organisms, which are a doublet oxygen molecule (O_2^*), which contains one additional unpaired electron[28]. The sources most commonly responsible for this are mitochondrial cytochromes and NADPH oxidases both in myocytes and in phagocytes[1]. In the mitochondrial cytochromes, the amount of superoxide produced is heavily debated, but the production is thought to be an unwanted byproduct of aerobic metabolism. However, the production of superoxide from NADPH oxidases is a very important and necessary function for inflammation and immune responses to infection. Certain cells, such as phagocytes, use this mechanism to produce superoxides, which can deliberately damage invading cells or infected/damaged cells. Thus, superoxides are a common radical in many organisms, however inability to manage these radicals, especially incidental production, may lead to systemic problems. Additionally, NADPH oxidases are also an important factor in vascular control of endothelial function, especially with exercise training[29], lending to the idea that these ROS are requisite for proper function.

Another oxygen molecule that can cause damage is the singlet oxygen, which is one oxygen atom with an unpaired electron. The production of this radical comes from both cytochromes in the cell as well as from UV light exposure[30]. Due to its high reactivity in this state, it is very short lived, but may react with a number of molecules or

atoms in close proximity to its location. Similar to superoxide, damage may be caused to cellular lipids, proteins, and DNA, which could alter cellular function.

Both superoxides and singlet oxygens have the ability to be reduced to hydrogen peroxide, usually by an antioxidant enzyme. Hydrogen peroxide is less reactive by itself than these radicals but could possibly be converted into other radical species in the presence of metals, demonstrated in the Fenton reaction (reaction 1) and Haber-Weiss reaction (reaction 2). Most importantly, this yields the presence of a hydroxyl radical (OH·), which may be a common source of cellular damage. This also tends to be very short lived due to its reactivity, but its occurrence is relatively high compared to other radicals.

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + \cdot OH + OH^- \text{ (Reaction 1)}$$

$$O_2^- + H_2O_2 \xrightarrow{Metal} \cdot OH + OH^- + O_2 \text{ (Reaction 2)}$$

Nitric oxide (NO) is a signaling molecule that is commonly synthesized in endothelial and neuronal tissues. It is produced from L-arginine by nitric oxide synthase, of which there are three forms: inducible (iNOS), endothelial (eNOS), and neuronal (nNOS). The most commonly known function is to promote local vasodilation in the endothelium to promote increases in local blood flow. While this molecule remains important for proper organismal function, NO can become reactive and be a source of RONS. NO may react with a superoxide molecule and become peroxynitrite (ONOO-).

This molecule has been noted to cause significant amount of damage to cell membranes, intracellular proteins, and DNA.

Antioxidant Defense System

In order to protect cells from damage from the aforementioned molecules, antioxidants are produced in many compartments in almost every cell type. Antioxidants exist in many forms and are vital in protecting the cell from excessive damage, since excessive levels of RONS may lead to irregular cell function or possibly cell death. The antioxidant system is very robust and has components mostly in the cytosol and mitochondria of the cell, to handle the RONS that are produced in these areas. Within the broad term of antioxidants, there are two subtypes: enzymatic and non-enzymatic.

Enzymatic antioxidants are molecules that facilitate the conversion of RONS to less-reactive or neutral species. These antioxidants are important for cell survival, as it determines the amount of RONS that can be neutralized, which attenuates damage related to OS. As with other enzymes, the amount of substrate that can be converted is largely dependent on the quantity of enzyme and the enzymatic activity. Thus, for cell survival, it is important to have enough antioxidant enzymes to quench the amount of RONS that are being produced.

There are many different kinds of enzymatic antioxidants, and typically use a metal as one of the primary catalysts to help reduce RONS. The three most common enzymes that act as antioxidants in the cell are Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPX).

SOD is a widely expressed antioxidant enzyme. Its main purpose is to reduce superoxide molecules into hydrogen peroxide and oxygen. It exists in three forms in human: SOD1 in the cytosol, SOD2 in the mitochondria, and SOD3 in the extracellular matrix. SOD2 contains manganese as its cofactor, while SOD1 and SOD3 contain copper and zinc as cofactors. CAT is a major enzyme that is vital for normal cell function. It is widely expressed in all mammalian tissues, is highly conserved, and is responsible for the breakdown of hydrogen peroxide into water and oxygen. Compared to GPX, another enzyme that breaks down hydrogen peroxide, CAT has a much lower binding affinity[31]. GPX reduces hydrogen peroxide into water and oxygen molecules, using two molecules of glutathione in the reduced form (GSH) and produces an oxidized glutathione disulfide (GSSG). There are eight known isoforms of GPX (GPX1-8). GPX1 is the most common form, existing in the cytosol of most mammalian tissues. These enzymes contain selenium, which is important for its function and regulation.

Additionally, there are three other enzymatic antioxidants, but they play a smaller role than the previous three. These antioxidants are thioredoxin, glutaredoxin, and peroxiredoxin. Thioredoxin (TRX) is a small, naturally occurring antioxidant that is found in the cytosol and mitochondria of most organisms and tissues[32]. It has two thiol sites that act to reduce disulfide bonds, creating an internal disulfide bond on TRX when oxidized[33]. Glutaredoxin (GRX) has similar characteristics to TRX in that it is responsible for reduction of disulfide bonds of cellular components, however, it uses a reduced glutathione molecule to catalyze the reaction[34]. Peroxiredoxin (PRX) is the third

major accessory antioxidant enzyme, and is mostly responsible for the reduction of hydrogen peroxide, lipid peroxides, or peroxinitrites, and may be reduced by TRX[35].

In addition to enzymatic antioxidants, there are non-enzymatic antioxidants that are responsible for helping to control the redox state. These include glutathione, uric acid, and bilirubin. Glutathione is the most abundant and sensitive antioxidant and is represented throughout most organisms and tissues in the cytosol. Glutathione exists in two states, reduced and oxidized (GSH and GSSG, respectively). GSH is a tripeptide that contains a thiol group that donates a hydrogen ion as its means of reduction; upon oxidation, two glutathione molecules combine and form a disulfide bond[36].

Uric acid is a naturally occurring molecule that is a byproduct of purine nucleotide metabolism, and is believed to be a large contributor to reduction of peroxides in the blood that form from the Fenton reaction at hemoglobin[37]. Interestingly, uric acid has also been shown to increase antioxidant production through the Nrf2-KEAP1 pathway (outlined below)[20].

Bilirubin is thought to be the product of heme catabolism, but its role as an antioxidant is lesser known. When heme is broken down by heme oxygenase 1 and 2 (HO1 – inducible, HO2 – constitutive), the end product is biliverdin. Biliverdin reductase is an enzyme that converts biliverdin to bilirubin, which can then act as an antioxidant[38]. Also of interest, HO1 is inducible by the Nrf2-KEAP1 pathway (outlined below)[39].

Antioxidant Production Signaling

Since antioxidants are vital to proper cellular function and organism survival, the regulation of production must be able to respond to any insult that increase ROS.

Production of antioxidants has been shown to occur in response to many oxidative insults, such as increased metabolism, hypoxia, UV/radiation exposure, and inflammation. Many of the molecules and enzymes are endogenously produced, so the increase in antioxidants is often due to increased transcription and translation of antioxidant genes.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a 605 amino acid 65-68 kDa transcription factor that is considered to be a 'master regulator' of antioxidant production and stage II detoxifying enzymes. It belongs to the basic leucine zipper family of transcription factors, in the Cap'n'Collar (CNC) subfamily. Nrf2 has seven domains, referred to as Nrf2-ECH homology (Neh). Each domain has binding capabilities with other molecules, which are still being researched, but appear to play a role in the activation, inhibition, or modification of Nrf2: Neh1 is a binding site that is associated with musculoaponeurotic fibrosarcoma proteins, (s-Maf) proteins, which allow for Nrf2 to exert its potential as a beta leucine zipper and initiate transcription of ARE-related genes; Neh2 is the main domain responsible for binding with KEAP1, and contains both DLG and ETGE domains that interact with tamtrack bric-à-brac (BTB) region on KEAP1; Neh3-5 contain motifs that aid in transactivation, binding necessary co-factors for transcription; Neh6 is believed to be involved in degradation pathways that involve the binding of b-transducin repeat-containing protein (b-TrCP); Neh7 has a binding site that allows for the binding of retinoid X receptor alpha (RXRa), but its physiological role remains unclear.

Nrf2 may translocate to the nucleus and dimerize with s-Maf. This dimer binds to antioxidant response elements (AREs) on DNA, which promotes the transcription of phase II detoxifying enzymes. There are more than 200 identified genes that respond in this manner, but some of the most important include HO1, SOD1/2, and NAD(P)H:quinone oxidoreductase 1 (NQO1). These antioxidants are important for a negative feedback loop to decrease the amount of oxidative stress.

Kelch-like ECH associated Protein 1 (KEAP1) is a 611 amino acid 69.7 kDa homodimer protein that was once referred as an inhibitor of NRF2. It exists in the cytosol of the cells, and is responsible for binding and sequestering Nrf2, which prevents Nrf2 from acting as a transcription factor in the nucleus. KEAP1 has three different functional regions: the broad-complex, tamtrack bric-à-brac (BTB) region, the intervening region (IVR), and the kelch region. The BTB domain is where KEAP1 proteins bind to create the homodimer. Additionally, there is the ability in this BTB domain to interact and bind Cullin-3, which is a E3 ubiquitin ligase, and is responsible for ubiquitination of Nrf2 for degradation through the 26S proteasome system. The IVR is the middle region on KEAP1 and contains a number of interacting cysteine residues; some of these cysteines play an important role in controlling the polyubiquination of Nrf2 or its release of Nrf2, potentially determining Nrf2's biological activity. The BTB domain also contains a cysteine residue (Cys151) that is thought to modify the Cullin-3 binding and/or activity of Nrf2 polyubiquination. The cysteine residues are thought to be sensitive to the redox status in the cell, and thus respond to oxidative stress through the cysteine residues. The kelch repeat region is the region that interacts and binds Nrf2. Nrf2 contains ETGE and

DLG motifs, each one binding to a kelch-repeat region of each of the KEAP1 molecules in the homodimer. The ETGE (hinge) has a stronger binding affinity than DLG (latch) and are often called the 'hinge' and 'latch' of the binding process with KEAP1. Each of these motifs contains critical cysteine residues that affect the binding affinity of Nrf2 to KEAP1. When RONS are present, they appear to modify the thiol groups on the cysteine residues, allowing for modulation of the binding characteristics of Nrf2.

This model of binding is referred to as the latch-and-hinge model and is one of the most widely accepted current theories for KEAP1 regulation of Nrf2 activity. According to this theory, the binding of the DLG motif to KEAP1 allows for proper alignment of Nrf2 to be polyubiquitinated by Cullin-3. This signals degradation of the Nrf2 molecules by ubiquitin proteasomes, turning over Nrf2 in unstressed states. However, if RONS are present to modify the cysteine residues at the IVR, then there is a conformational shift that does not permit binding at the 'latch' site. In this state, Nrf2 is not available to be biologically active, as it remains bound to KEAP1 at the ETGE motif, but it is not properly aligned to be ubiquitinated for degradation. This state may be the most stable confirmation for Nrf2 but remains contentious. Upon further stress from RONS, the cysteine residue at the ETGE binding site is modified and facilitates the release of Nrf2 from KEAP1. It is then that the Nrf2 molecule may translocate into the nucleus and go through the process of signaling translation of ARE-genes. This form of activation is now referred to as the 'canonical' activation of the KEAP1/Nrf2 system.

In the 'non-canonical' form of KEAP1/Nrf2 activation, p62 is thought to control Nrf2 release from KEAP1 as well. This autophagy related protein, p62, has been noted to

interact with KEAP1 on the same sites as NRF2 binding sites. Nrf2 has a 30 fold higher binding affinity than p62 on KEAP1, but the binding affinity of p62 can be increased by phosphorylation at serine at the 351 location (5 fold less than Nrf2)[40]. The current theory suggests that as p62 levels increase, which may occur when autophagy is inhibited, p62 may bind to KEAP1 at the DLG domain and possibly the ETGE domain in high enough concentrations, freeing up NRF2 for translocation into the nucleus (similar to the latch-and-hinge model). As previously mentioned, the binding affinity of p62 increases when phosphorylated at serine 351 location, which has been observed to happen with mammalian target of rapamycin complex 1 (mTORC1). Similarly, mTORC1 has been noted as slowing autophagy through inhibition of ubiquitin ligase kinase 1 (ULK1), explaining the buildup and subsequent phosphorylation of p62. Thus, it appears that mTORC1 may be partially responsible for the non-canonical activation of the KEAP1/Nrf2 pathway.

Additionally, since p62 is responsible for binding tagged proteins to the autophagolysosome during autophagy, some studies have examined and noted KEAP1 degradation in p62 overexpression, as well as a KEAP1 accumulation during p62 deficiency. Accumulation of p62 can occur when autophagy is inhibited; typically, this occurs when autophagy-related proteins (Atg's) are inhibited (typically atg5 and atg7). In studies that knock down atg7, a consequential accumulation of p62 occurs, leading to KEAP1 degradation, Nrf2 activation, and downstream antioxidant production. This, as well as the mTORC1 phosphorylation of p62, demonstrates that p62 may be responsible

for alternative liberation of Nrf2 from KEAP1 as well as the subsequent degradation of the KEAP1 molecule.

Once Nrf2 has been released from KEAP1 by canonical or non-canonical activation, it is able to translocate to the nucleus. Since it is a transcription factor, it must cross the nuclear membrane to bind to DNA and promote transcription. The full mechanism of this facilitation is not well known; for example, some studies suggest that modifications, such as phosphorylation, occur once Nrf2 is in the nucleus, prohibiting Nrf2 from leaving the nucleus. However, it is widely accepted that Nrf2 must interact and bind with the aforementioned s-Mafs in order to bind to the ARE-motifs, signaling antioxidant production.

Exercise and Antioxidant Production

As previously mentioned, exercise is a stress that causes a change in the antioxidant defense system. It has been noted that ROS increase during exercise of sufficient intensity and duration, and consequentially leads to signaling of antioxidant production. The source of ROS during exercise is not fully understood, but current theories suggest increased aerobic metabolism or increased NADPH oxidase activities are at least partially induced.

Originally, it was thought that superoxides and singlet oxygen radicals were produced in the mitochondria due to mishandling of the oxygen by cytochromes. It therefore stood logical that as aerobic metabolism increased the amount of oxygen being utilized in the mitochondria, the number of superoxides and singlet oxygen radicals would increase. However, some studies suggest that this mechanism may not hold true,

and that cytochrome ROS production within the mitochondria may not increase to the extent previously thought. While it is well documented that ROS increase with exercise, it may not be due to an increase in overall metabolism. Instead, some evidence now points to increased NADPH oxidase activity[41].

NADPH oxidases (NOX) are membrane-bound enzymes, often extracellular-facing, that are responsible for producing superoxides and $NADP^+$ from oxygen and NADPH. This is the process used in phagosomes as a way of causing damage to target cells, which is vital for responses to infection. However, many cells contain oxidases for various purposes. Some of the most studied NOXs are in endothelial tissues, and are responsible for proper vascular function, particularly during and responding to exercise. Research in the past decade has elicited its importance, especially leading to diseased states, such as cardiovascular disease. In this case, NOX can be problematic, as increased superoxide production from increased NOX activity causes peroxinitrite production when combined with NO production[42]. This scenario leads to less NO signaling required for vasodilation, which may lead to cardiovascular dysfunction if repeated over time. However, NOX a larger beneficial physiological role contributing to vasodilation under certain circumstances as its product superoxide is often reduced to hydrogen peroxide, which can translocate to the smooth muscle cells and cause hyperpolarization-related vasodilation[43].

However, NOX plays a larger beneficial physiological role, with most of the beneficial effects exerted by NOX2 and NOX4 through ROS cell signaling. Production of ROS from NOX2/4 act as cellular signals in the adjacent smooth muscle cells to increase

the activity of p38 MAPK and AKT signaling[44], which leads to robust cell survival responses, particularly protecting against inflammatory stress. These NOXs respond to a number of molecules, but most importantly respond to shear stress, HIF-1, and angiotensin II[45]. These oxidases play a large role in short- and long-term vascular function which is vital for exercise adaptations; additionally, these ROS-dependent responses that NOX induces may play a large role in antioxidant defenses as well, which may include the Nrf2/KEAP1 pathway. While it may seem counter-intuitive to have enzymes in many tissues that are responsible for producing ROS both during exercise and at basal states, it has become clear that their overall function is to stimulate ROS-dependent cell survival pathways.

Regardless of source of ROS, the intensity of exercise appears to be the largest determinant of oxidative stress[9]. Typically, for aerobic activities, a moderately high intensity, when there is high oxygen uptake by the skeletal muscle, appears to illicit more ROS-related damage. If the intensity is too high, the duration of exercise may not be long enough to accumulate a large dose-response. However, at moderately high intensities, ROS may accumulate over a long duration of time[10] and thus, duration is an important consideration for ROS production.

With increased aerobic exercise training comes robust adaptations to exercise. In particular, there are changes that occur in the redox system that promote antioxidant production. This helps protect cells against future insults of ROS, potentially mitigating the negative effects associated with ROS production. There are changes to almost every aspect of the redox system, some are well understood, while others are being investigated

as new areas. Many of these changes have been promoted as beneficial in managing or preventing diseases such as neurogenerative diseases, cancer, diabetes, cardiovascular disease, and more.

The most notable changes that occur in response to aerobic exercise training are increases in SOD, GPx, and CAT[46]. These responses generally differ slightly based on the type of tissue; for example, highly oxidative muscle fibers tend to create more antioxidants in response to training, whereas less oxidative fibers tend to increase to a lesser degree[10]. Production of these antioxidants typically come from a number of signaling pathways, a built in mechanism to ensure activation due to a number of stressors, which include Nrf2, NF-kB[47], PPARs[22,48], and MAPK pathways. Nrf2 has recently been implicated as more important in antioxidant-specific adaptations and is highly responsive to the redox status of the cell. The other cell factors are more responsive to inflammation, nutritional stress, or other forms of stress, and have a less-specific antioxidant response.

While the antioxidant response to endurance training in the muscle has been widely studied, much less is known about the regulation of antioxidant production signaling, which appears to be largely controlled by Nrf2 and KEAP1. As of late, more research has been done to address the roles of these two molecules, but many results are conflicting and lead to questions about methodological differences as well as the importance of key metrics.

Exercise and KEAP1-Nrf2 Pathway

Since Nrf2 is considered the 'master regulator' of antioxidant production, Nrf2 has been the focus of many studies evaluating cellular redox responses. Initial studies evaluated changes in Nrf2 after training compared to before training. Results using this design showed a mild increase in certain tissue types, but the correlation between Nrf2 levels and antioxidant levels were inconsistent and lacked strong evidence of a true causal relationship.

One of the first studies attempting to examine Nrf2 changes to exercise was by Asghar and colleagues in 2007[49]. Their previous studies had shown that oxidative stress decreased levels dopamine receptors (D1) and decreased associated G-protein activity[50]. As a continuation of their work, the purpose of this particular study was to examine the effects of exercise on oxidative stress management and hopefully see a restoration in D1 receptor number and activity. Since Nrf2 is a promoter of oxidative stress, they examined nuclear Nrf2 levels, but not whole cell Nrf2, to gauge the hormetic redox response. Adult Fisher rats (23 months old) were exercised on a treadmill for 6 weeks, 60 minutes at 15m/min at a 15-degree grade for 5 days each week. At the end of the 6th week, rats were sacrificed 48 hours after the last exercise session, and the proximal renal tubule (PRT) was removed for analysis. In the PRT of exercise rats, compared to sedentary rats, there was: a decrease in malondialdehyde, suggesting a decrease in oxidative stress damage; an increase in SOD activity and quantity, suggesting increased antioxidant capacity; and an increase in nuclear Nrf2, suggesting an increase in antioxidant production signaling. While there were many other measures and focal points of this study, this was one of the

first to examine exercise's effects on Nrf2 signaling. The data suggest that exercise promotes Nrf2 activation and subsequent antioxidant production. However, there are many limitations and variables that remain unknown. This study did not examine the effects of exercise on total Nrf2 levels or the pre- to post-training changes in KEAP1, which is a major factor in regulating Nrf2. Additionally, these changes were only observed in the PRT, which may not correlate to changes found in other tissues. However, being the first study to examine the effects of exercise on Nrf2 is important as it led to a demonstrated response in Nrf2 activation and possible mediation of antioxidant response systems.

A couple of years later, this team, this time led by Liza George, conducted a similar study where Fischer rats were exercised on a treadmill at 12 m/min and 15 degree grade for 60 min, 5 days per week, for 12 weeks in total[51]. In addition to longer exercise training, there were two ages of rats (3 months and 21 months old) undergoing exercise training, with age-matched sedentary controls, for four total groups. By doing this, the team was able to assess the aging-based differential response to exercise. Again, the focus of their study was on PRT tissues and the dopamine receptor responses in the tissues; however, they again measured nuclear Nrf2 (but not whole cell) and downstream antioxidants and activity. The results demonstrated similar findings to their previous study: decreased malondialdehyde (MDA), increased nuclear Nrf2, and increased antioxidant capacity. However, it appeared that the older rats had greater levels of MDA, increased levels of nuclear Nrf2, and different antioxidant responses. The SOD response between adult and old rats was fairly similar; however, the HO1 response was much

higher in exercised old rats than in younger groups. The increased signaling (increased Nrf2) in the elder rats suggests a compensatory mechanism for either higher damage (increased MDA) or potentially diminished capacity to manage the damage and stress through other means. The differential response in antioxidants suggests that there may be an alteration in the translational or post-translational regulation of ARE genes that are the target of Nrf2. However, the authors did examine NF-kB in this study, as it has overlap with SOD1 and HO1 genes. They found a similar pattern in NF-kB as they did with Nrf2, suggesting that the two transcription factors respond similarly to exercise. However, this did not fully explain the altered pattern of HO1 expression. Similarly to the first study, there was no inclusion of whole cell Nrf2 or any KEAP1 measurements. The study provided evidence for an age-related response, which is important for antioxidants' connection to aging-related illnesses.

In 2012, Gounder et al. followed a similar approach to this exercise, oxidative stress, and aging model using cardiomyocytes from C57/BL6/SJ mice[17]. Young (2 months) and old (23 months) mice were exercised for 6 weeks of 50 minutes per day at 10m/min and 7% grade. The cardiomyocytes were assessed for nuclear Nrf2, a number of proteins that are part of ARE-related genes, and oxidative stress markers. There was a noted decrease in Nrf2 and increase in oxidative stress in sedentary older mice compared to sedentary young mice. After exercise training, it appeared that most of the ARE-related antioxidants were elevated compared to sedentary controls, but that younger mice had a larger increase compared to older mice. In addition, nuclear Nrf2 was lower for older mice than younger mice both pre- and post-training; the difference between the two

ages was less pronounced after exercise training. This study provides additional evidence that Nrf2 has altered responses in older mice compared to younger mice, but that exercise tends to increase its nuclear translocation.

In 2016, Merry and Ristow trained mice for 6 weeks, 5 times per week, on a treadmill[52]. The results demonstrated that mRNA for Nrf2 increased significantly after 6 weeks of training, and mRNA for Nrf2 targets also increased. It is important to note that at least 36 hours passed since the last training session, so these increases likely are representative of an elevated basal state. Additionally, inclusion of a Nrf2 knockout model undergoing the same training demonstrated less overall down-stream protein production. This suggests that Nrf2 is a required part of this pathway, and plays a valuable role in producing antioxidants. However, this study did not measure Nrf2 protein levels, cellular subfractions, or Nrf2 activation. Nrf2 mRNA only suggests a transient signal to provide more Nrf2; given its short half-life (20-200 minutes) this enhanced signal to increase Nrf2 activation may have occurred but remains speculative. This study did not examine many regulatory points nor final antioxidant protein level, but it is one of a few studies to assess NRF2 in a chronic exercise training model.

Since these initial studies, less work has been done on the specific changes in the pathway regulation, but some studies still include Nrf2 in the measurements as a secondary purpose to a pathological condition. In 2019, Yu et al. published their study examining exercise training in female Sprague-Dawley rats, with either high fat diet or control diet[53]. The exercise was 30min per day on a rotarod for 9-12 weeks, and hamstring and gastrocnemius muscles were examined for Nrf2 and KEAP1 levels using

western blot analysis. This is significant as this is the first known study that examined KEAP1 protein changes due to exercise training. The results demonstrated an increase in Nrf2 levels as a result of exercise training, regardless of diet. Additionally, there was a slight but significant reduction in KEAP1 due to exercise training, regardless of diet. When examining SOD1, a target of Nrf2, there was increased protein levels in rats that were exercised, regardless of diet. The results together suggest that exercise training modulates the Nrf2 and KEAP1 levels, which ultimately impacts the levels of antioxidants produced. While there was no direct translation of a Nrf2/KEAP1 ratio, it appears that this measure may change as a result of training, and that this ratio change may correlate to the changes in antioxidant production. While the rotarod training may be a form of exercise, it may cause a slightly different response than exercise training on a treadmill, due to its increased motor coordination utilization and different muscle recruitment patterns. This appeared to be enough of a stimulus to cause a response but use of a treadmill may lead to slightly different results. These results add to the emerging body of evidence that exercise alters the KEAP1/Nrf2 pathway, and the ratio of these two proteins that affect the antioxidant capacity of the cell.

A recent study by Rahimi et al. in 2021 was another study to examine KEAP1 protein in a type-2 diabetes mouse model[54]. For this study, 35 C57BL/6 mice were used, in seven different groups (n=5/group). These mice were induced with type 2 diabetes by inactivity and diet supplementation. Then, mice were treated with combinations of a salvia blend, salvia, metformin, and exercise. For our purposes, the control, diabetes, and exercise+diabetes groups are the ones of interest. The particular tissue of interest was

liver, for all dependent variables. At basal levels, the diabetes group had much less Nrf2 (whole cell) and much higher KEAP1 levels than control mice. This indicates that the upstream redox signaling molecules are altered in diabetes mellitus. While there was no diabetes negative with exercise control group, the exercise component looked at diabetes positive and sedentary as the control group with diabetes and exercise as the intervention group. The exercise consisted of 8 weeks of exercise, 5 days per week, 45 minutes per session, training at 0 degree grade, with an initial speed of 10 m/min. Speed was then increased 3 m/min until the final session was at 25 m/min. The exercise+diabetes group showed a marked recovery in Nrf2 and KEAP1 protein levels, similar to that of the original control group (although not directly tested with a statistical measure). The results from this study indicate that Nrf2 increases and KEAP1 decreases in response to exercise training over 8 weeks. However, it is important to note that these are in diabetes mice, which means that exercise plays a restorative function in these proteins, and does not necessarily represent changes that would occur in otherwise apparently healthy mice. Additionally, these represent liver changes, and not necessarily changes that would occur in other tissues. While this study does not answer many questions regarding exercise adaptations in the full Nrf2/KEAP1 adaptation, including cellular localization, it is one of few studies addressing KEAP1 changes in response to exercise training, and reflects findings that are similar to those of Yu et al, in 2019.

Although chronic exercise training has been the primary focus of the few studies examining the KEAP1/Nrf2 pathway, a few studies have attempted to assess the acute effects of exercise. The first study focusing on the KEAP1/Nrf2 pathway with exercise

was by Muthusamy et al. in 2012. Exercise was used as an acute stress in this study, with two consecutive days of 60 minutes on a treadmill at 14 m/min and 10% grade. This methodology was performed both in wild-type and $Nrf2^{-/-}$ knockout mice. Mice were sacrificed immediately upon cessation of the second exercise session, and heart tissue was harvested for analysis. Exercise caused an increase in Nrf2/ARE binding activity, and a large increase in nuclear Nrf2 protein levels; change in whole-cell Nrf2 was not measured. Upon examining downstream antioxidants (protein levels), the wild-type mice appeared to increase enzyme production for some, but not all Nrf2 target genes with exercise There were significant increases in G6PD, GCLm, and HO1, but not in catalase, GCLc, NQO1, GSR, or GPX1. $Nrf2^{-/-}$ mice demonstrated overall lower levels of antioxidants at baseline, and typically a reduction in most enzymes after exercise. However, HO1 was higher in baseline than wild-type controls and had an increase after exercise. This demonstrates that there may be regulation of this gene outside of Nrf2; in some studies, NF-kB has been suggested to increase HO1 production as well as Nrf2[55].

The work of Li et al. followed in 2015 to examine the duration effect of exercise[56]. For their study, they used C57BL/6J mice exercising for a single one- or six-hour session at 20m/min and 5% grade and harvested whole hindlimb skeletal muscle. From these results, they found that Nrf2/ARE binding activity (through a transcription factor activation kit) was increased significantly after the 6-hour bout of exercise, but not after the one-hour bout of exercise. Additionally, when examining Nrf2 protein levels, there were significant increases in protein levels found in the nucleus after 6 hours, but not after 1 hour. KEAP1 was measured as well during this study and demonstrated a

significant increase in protein levels after 6 hours of exercise, and a modest but statistically insignificant increase after 1 hour of exercise. Downstream antioxidant targets of Nrf2 (GCLc, GCLm, SOD1, SOD2, CAT, HO1) all showed significant increases in mRNA levels after exercise, with longer duration leading to higher increases.

This study is mostly in agreement with the work from Muthusamy, however, there remain questions about the duration. Muthusamy did two 1-hour exercise sessions over two days and found significant changes in the results, but Li found no differences immediately after a 1- hour exercise training session. It seems plausible that the back-to-back days of Muthusamy was enough to see residual changes from the prior day as well as a preconditioning effect. Li suggests that even at a higher speed than Muthusamy's work, 1 hour is insufficient, but 6 hours was more than enough to elicit a response.

The only other study to examine the effects of duration came a year later in 2016, when Wang et al. performed a study examining the Nrf2 response in ICR/CD-1 mice exposed to different acute exercise bouts (45, 90, 120, or 150 minutes). Using gastrocnemius and quadriceps muscles, a linear increase in Nrf2 mRNA was seen from 45 up to 120 minutes, with a slight decrease from 120 to 150 minutes. The protein levels of Nrf2 did not have a significant increase at 45 minutes of exercise but appeared to have an increased and plateaued response in protein levels after 90 minutes. Together, these results may suggest that during exercise, there is a signal to make more Nrf2, and that signal continues to increase, making more Nrf2 mRNA; once the Nrf2 has increased, the level of mRNA begins to come back down. The protein levels of SOD1 did not change throughout any duration of exercise, whereas SOD2 followed a similar response to the

Nrf2 protein levels (plateauing after 90 minutes). While this demonstrates the importance of Nrf2 in producing protective antioxidants, there are a number of pieces missing from this study. There is no observation of KEAP1 which may describe the regulation of Nrf2, and there was no nuclear fraction measure of Nrf2 or its binding activation. Compared to the other studies, it does appear that less than 60 minutes potentially causes an insignificant change in Nrf2 activation. These findings suggest that 90 minutes is sufficient duration to find Nrf2 changes in a single bout of exercise (if at the proper intensity). However, as with many exercise studies, repeated bouts of exercise will alter the antioxidant levels, so it may not be necessary to do as extensive duration to see some of these physiological changes (also dependent on intensity and age of the subjects).

Since these three aforementioned studies, not much work has been done on understanding the relationship between duration and Nrf2 response. However, a few studies have also been conducted to examine the effects of exercise intensity on the Nrf2 pathway.

In 2017, Done, Newell, and Traustadottir used human subjects to examine the intensity effect of exercise on the Nrf2 pathway. The study was a cross-over design using two 30 min exercise bouts on a cycle ergometer, one being High Intensity Interval Training (HIIT; 90% VO_2max for 1 min and 60% VO_2max for 2 minutes, after a graded warmup), and the other was a continuous workload bout (CW; 70% VO_2max). Blood was taken before, immediately after, and 30 minutes after the exercise session; this blood was then used to isolate peripheral blood mononuclear cells (PBMCs), which were used for Nrf2 analysis. From before to after the exercise session, whole cell Nrf2 was not

significantly affected in either HIIT or CW groups. When examining nuclear Nrf2, there was a time effect after 30 minutes post-exercise, but no significant effect in trial type. In this study, one of the few done in human subjects, 30 minutes of moderate-to-high intensity exercise appeared to be sufficient to lead to Nrf2 nuclear translocation, regardless of exercise type. However, whole cell Nrf2 was not changed; this may be a function of lack of duration, as outlined before, or a lack in measuring regulatory mechanisms. KEAP1 has a strong influence on sequestering and release of Nrf2 and should be factored in. No direct measures of protein levels were measured to assess the downstream effects of the pathway, but some enzyme activities were measured, with no significant differences.

Since Nrf2 is a transcription factor, it has been more widely accepted that the nuclear location of Nrf2 is more biologically relevant than whole cell levels. However, most studies just examine one measure or the other, which leads to a tough interpretation. Many of the studies done thus far have used the Nrf2 TransAm Activation kit, which is a kit aimed at determining transcription factor activation, by pulling down Nrf2 bound to DNA and labeling with a Nrf2-HRP antibody. While this may be a quicker way to measure Nrf2 activity, the lack of cytosolic Nrf2, nuclear Nrf2, and cytosolic KEAP1 leaves a fundamental gap in understanding the pathway's responses to exercise.

Since KEAP1 is considered to be an inhibitor of Nrf2's biological availability, as well as the target of the E3 ligase, cullin-3, which aids in the degradation of Nrf2. Thus, KEAP1 plays an important regulatory role in the relevance of Nrf2. While studying Nrf2 in isolation could lead to understanding, no known research to date has evaluated the

changes in KEAP1 levels in response to regular exercise training, which is important to consider.

If cytosolic KEAP1 levels increase and Nrf2 increases, this might suggest an adaptation to raise the NRF2 level for a stress condition. If the ratio decreases – that is KEAP1 levels are not matched to NRF2 then this might be a transitionary phase to change the level based on the stress. Or it might mean that there was a need to down regulate the antioxidant protection. If levels go down this might suggest less protection when an OS event occurs.

CHAPTER III

METHODOLOGY

Animal Model

For this study, both C57BL/6 wildtype mice and a LC3-transgenic mouse model, which is bred on a C57BL/6 strain, were used. Mice had *ad libidum* access to water and food, consisting of a standard chow diet. Mice followed a typical 12:12 light:dark cycle. Mice were divided into sedentary and chronic exercise groups and beginning intervention between 8 and 12 weeks of age. The sedentary group consisted of 9 mice, while the exercise group contained 14 mice. Sedentary mice were sacrificed at the same time as exercise mice, with no intervention over the same time course. All mice were bred from the same distributor, and internally maintained from breeder rotation.

The University of North Carolina Greensboro Institutional Animal Care and Use Committee (IACUC) approved all procedures and experiments prior to animal testing.

Exercise Training

Exercise training occurred on a rodent treadmill with a 12-lane divider. The divider was outfitted with a shock grid at the rear of each lane to encourage mice to run. Additionally, mice were deterred from the back of the lane with a bottle brush gently nudging their tail from underneath the divider when needed.

Mice exercised 5 days per week (Monday-Friday) between 0800 and 1200 (during the light cycle) for 8 weeks, following the protocol outlined in Supplement 1. The

protocol was designed to increase speed and/or grade every 2 weeks and be about 65% of VO_{2max} for mice[57].

Sacrifice and Tissue Harvest

At the end of 8 weeks, mice were sacrificed at 48 hours after the last training session to evaluate basal biological marker levels. Mice were anesthetized using sulforaphane, followed by cervical dislocation. Gastrocnemius, soleus, superficial (white) quadriceps, and deep (red) quadriceps were harvested into 1.5 ml microcentrifuge tubes and flash frozen using liquid nitrogen. Samples were then stored at -80 ^{o}C for future analysis.

Upon preparation, samples were thawed, and approximately 30mg of quadriceps separations or gastrocnemius tissue were combined with 500 µL of lysis buffer (10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.5mM $MgCl_2$, 1mM dithiothreitol, 0.1mM phenyl methylsulfphonyl fluoride, and 1% Triton-X; pH 7.9) and homogenized using a QIAGEN TissueLyser LT Tissue Homogenizer (Hilden, Germany). Since soleus muscles did not have 30mg of tissue, both left and right soleus were combined (12-15mg total) and homogenized in 250uL of buffer. The homogenate was then centrifuged at 5200 rpm 2416 x g for 5-6 minutes at 4 degrees Celsius. The supernatant was collected as the cytosolic fraction, while the pellet was saved to be used as the nuclear fraction. The nuclear pellet was washed with lysis buffer to rinse away cytosolic contaminants, and then the nuclear pellet was resuspended in 500 µL nuclear lysis buffer (20mM HEPES, 420mM NaCl, 0.1mM EDTA, 25% glycerol, 1mM dithiothreitol, and 0.5mM phenyl

methylsuphonyl fluoride; pH 7.9). This nuclear fraction underwent another centrifuge cycle at 5719 x g for 10 minutes at 4 degrees Celsius.

Bradford Assay

In order to determine the protein content in each sample, 30ul of sample was combined with 150ul of Bradford Reagent (50mg Brilliant Blue, 50ml methanol, 100ml of 85% H_3PO_4, 850ml dH_2O). A standard curve using bovine serum albumin was created with concentrations ranging from 0-2000ug/ml. Samples and standards were pipetted into a 96 well plate and read at 595nm wavelength using a Gen5 BioTek Microplate reader (BioTek; Winooski, VT, USA). All samples were determined at least in duplicate.

Western Blot Analysis

Cytosolic and nuclear subfractions were thawed and analyzed using BCA analysis for protein content. From the results of the Bradford Assay, a volume delivering an estimated 30 ug of protein combined with 6x Laemmli Sample Buffer was boiled for 10 minutes and then loaded in each well of a 10-well 4-12% Bis-Tris graded gel (ThermoFisher; Waltham, MA, USA). Additionally, MagicMark XP Western Protein Standard (ThermoFisher; Waltham, MA, USA) was loaded into the first lane of each gel for molecular weight comparisons. Using MOPS running buffer in a mini-gel NuPage gel apparatus (ThermoFisher; Waltham, MA, USA), gel electrophoresis was performed for 55 minutes at constant 200V. The proteins were then transferred to a PVDF membrane (ThermoFisher; Waltham, MA, USA) using the same NuPage apparatus with a transfer sandwich apparatus for 30 minutes at constant 15V.

After transfer of the membrane was complete, the rest of the membrane was blocked using a 5% bovine serum albumin solution. Primary antibodies were used to identify the protein of interest in a 1:1,000 dilution in 5% BSA. The primary antibodies (anti-KEAP1, anti-Nrf2, anti-HO1, anti-SOD1, and anti-GAPDH) were prepared all in 5 mL of 5% BSA solution, each at a 1:1000 dilution. After a one-hour incubation in the primary antibody solution, the membrane was washed with 1X Tris-Buffered Saline, 0.1% Tween® 20 Detergent (TBST) for 10 minutes.

Using a GE Amersham Typhoon 5 imager (GE; Boston, MA, USA), blots were imaged using five different laser/filter combinations (488/Cy2LP, 532/Cy3LP, 635/Cy5LP, 685/IR short, 785/IR long; see appendix 2 for laser/filter/fluorophore compatibility). KEAP1, Nrf2, SOD1, and HO1 levels were normalized to GAPDH levels using ImageJ (NIH; Bethesda, MD, USA).

Statistical Analysis

Multivariate analyses were conducted in SAS (SAS; Cary, NC, USA), while multivariate normality, Pearson correlations, and graphs were all performed in RStudio (version 1.1.383, running R version 4.0.3, using packages tidyr, dplyr, MVN, and ggplot2). The a priori alpha level was set at 0.05. A repeated-measures multivariate analysis of variance (rm-MANOVA) was used to test the main effects of exercise, the within-subjects effect of muscle, and the interaction effect of exercise and muscle. Pearson correlations were used between nuclear Nrf2 and downstream antioxidants, as well as the total and cytosolic Nrf2/KEAP1 ratios with downstream antioxidants.

CHAPTER IV

RESULTS

Multivariate Normality

Since dependent variables (proteins) were expected to be a part of the same pathway and have similar responses in exercise training, Pearson correlations were performed between each protein to determine the degree of correlations between dependent variables. In many cases, high degree of collinearity between dependent variables warrants multivariate analyses. Results of the correlation matrix are shown in table 1, and demonstrate many significant correlations between outcome measures, suggesting multivariate analyses may be the best analysis technique.

*Table 1. Correlation table of protein dependent variables, regardless of muscle group. *denotes significance of $p < 0.05$*

	Nuc. Nrf2	Cyt. Nrf2	HO1	SOD1
KEAP1	r = 0.073 p = 0.493	r = 0.604 p < 0.001*	r = 0.248 p = 0.018*	r = 0.454 p < 0.001*
Nuc. Nrf2		r = 0.192 p = 0.070	r = 0.135 p = 0.209	r = 0.245 p = 0.020*
Cyt. Nrf2			r = 0.397 p < 0.001*	r = 0.335 p = 0.001*
HO1				r = 0.348 p < 0.001*

Table 2. Correlation matrices of dependent variables (proteins) by muscle group using Pearson correlations.

		Nuc. Nrf2	*Cyt. Nrf2*	*HO1*	*SOD1*
Gastroc.	KEAP1	r = -0.125 p = 0.57	r = 0.649 p < 0.001*	r = 0.444 p = 0.03*	r = 0.319 p = 0.14
	Nuc. Nrf2		r = 0.078 p = 0.72	r = 0.053 p = 0.81	r = 0.189 p = 0.39
	Cyt. Nrf2			r = 0.418 p = 0.047*	r = 0.335 p = 0.12
	HO1				r = 0.020 p = 0.93
Soleus	KEAP1	r = 0.497 p = 0.02*	r = 0.833 p < 0.001*	r = 0.501 p = 0.02*	r = 0.250 p = 0.26
	Nuc. Nrf2		r = 0.405 p = 0.06	r = 0.198 p = 0.38	r = 0.338 p = 0.12
	Cyt. Nrf2			r = 0.447 p = 0.04*	r = 0.179 p = 0.42
	HO1				r = 0.274 p = 0.22
Superficial Quad	KEAP1	r = 0.188 p = 0.39	r = 0.731 p < 0.001*	r = 0.634 p = 0.001*	r = 0.321 p = 0.13
	Nuc. Nrf2		r = -0.134 p = 0.54	r = -0.120 p = 0.59	r = 0.071 p = 0.75
	Cyt. Nrf2			r = 0.654 p < 0.001*	r = 0.644 p < 0.001*
	HO1				r = 0.311 p = 0.15
Deep Quad	KEAP1	r = -0.081 p = 0.72	r = 0.624 p = 0.002*	r = 0.303 p = 0.17	r = 0.462 p = 0.03*
	Nuc. Nrf2		r = 0.406 p = 0.06	r = 0.115 p = 0.61	r = 0.076 p = 0.73
	Cyt. Nrf2			r = 0.687 p < 0.001*	r = 0.469 p = 0.03*
	HO1				r = 0.418 p = 0.05

Correlation matrices separated by muscle group revealed less overall correlation between dependent variables, likely due to decreased sample size, but illustrate that multiple dependent variables are still correlated with each other and warrant multivariate analyses.

Normality Testing

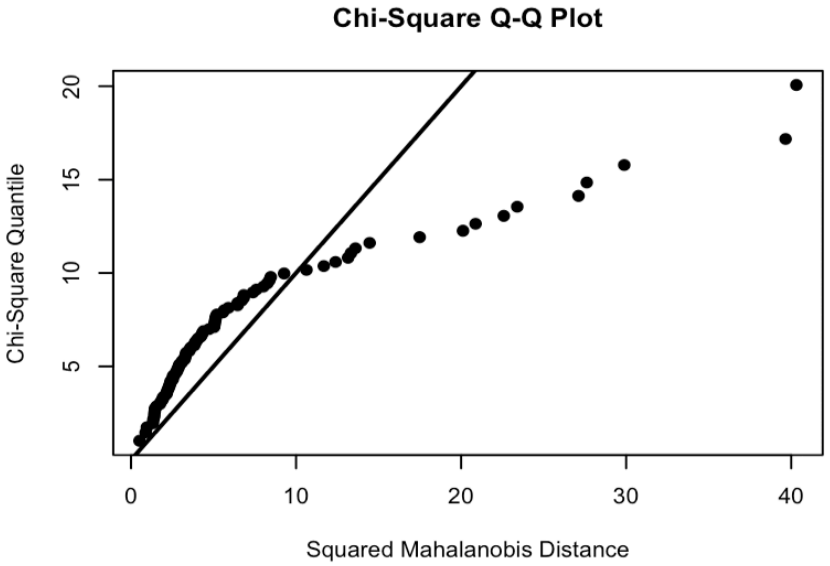


Figure 2. Multivariate Q-Q plot for normality of untransformed data.

Mardia tests for multivariate skewness and kurtosis both showed violations of multivariate normality ($p < .001$). Additionally, Shapiro-Wilk tests for univariate normality showed violations in univariate normality in all dependent variables ($p < .001$). Examination of the Q-Q plot (Figure 1) reveals that there is a large skew in the data. Examining the histograms (Figure 2) for each dependent variable in each group, it appears that there are some variables with positive skew and likely present as outliers in the data.

Table 3. Multivariate tests for skewness and kurtosis for untransformed data

Test	Statistic	P Value
Mardia Skewness	368.341	3.16e-57*
Mardia Kurtosis	15.494	0*

Table 4. Univariate Tests for normality of untransformed dependent variables.

Protein	Shapiro-Wilks	P value
KEAP1	0.7586	<0.001*
Cyt. Nrf2	0.7561	<0.001*
Nuc. Nrf2	0.8543	<0.001*
SOD1	0.8594	<0.001*
HO1	0.9268	<0.001*

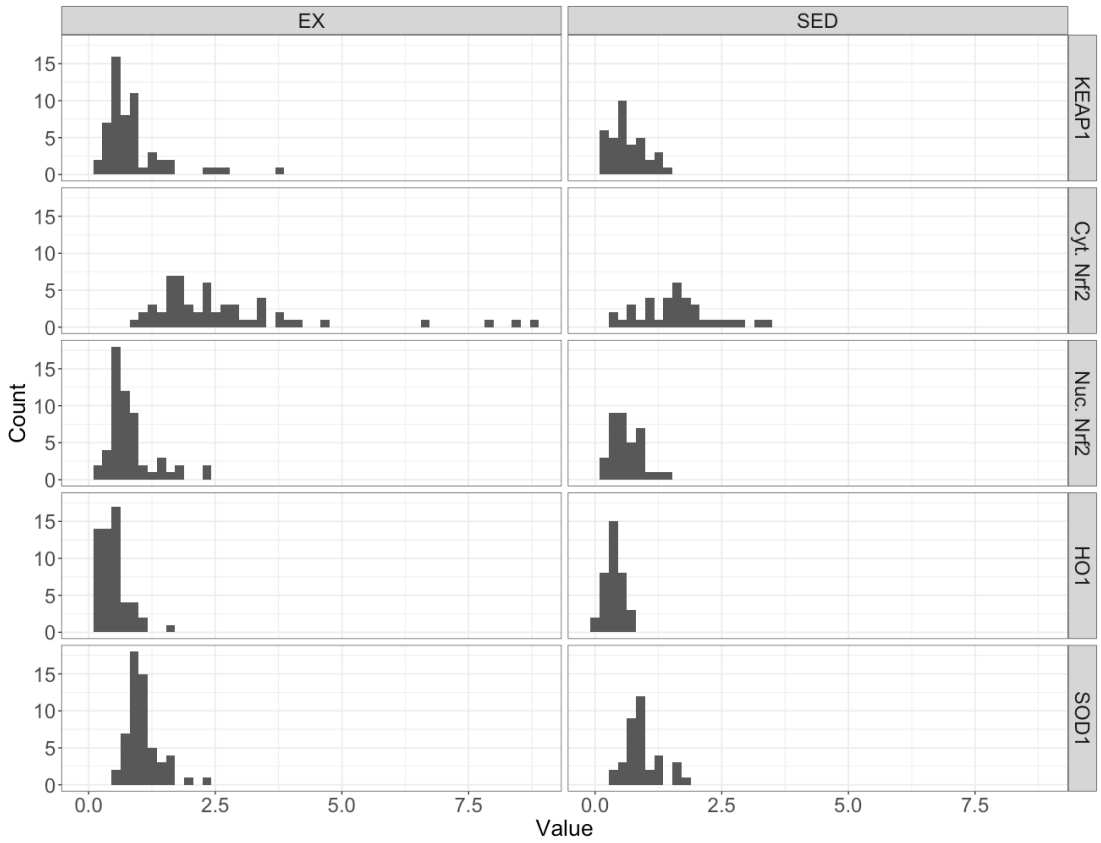


Figure 3. Histograms of protein levels, normalized to GAPDH, separated by exercise/sedentary group for untransformed data.

Table 5. Univariate normality measures for untransformed dependent variables. Acceptable skew values were expected to be within -2 to +2; kurtosis values expected in the range of -7 to +7.

Protein	N	Mean	Std.Dev.	Skew	Kurtosis
KEAP1	92	0.7826292	0.5630070	2.620191	9.496155
Cyt. Nrf2	92	0.7375909	0.4195829	1.705967	3.666213
Nuc. Nrf2	92	2.2640429	1.5014878	2.416328	7.127872
SOD1	92	1.0168997	0.3441429	1.104644	1.726374
HO1	92	0.4360454	0.2542988	1.879283	5.926578

Data Transformation

Since all dependent variables were suggested to violate normality, each variable was transformed using a natural logarithm. Log transformation is a common practice among biological samples where observations are all positive but follow a log-normal distribution pattern; after log transformation, the distribution pattern resembles a normal-distribution, allowing for statistical tests to be conducted. After retesting for normality, multivariate normality analysis still showed violations in multivariate skewness, but not kurtosis. Univariate analysis showed normality in all observed dependent variables.

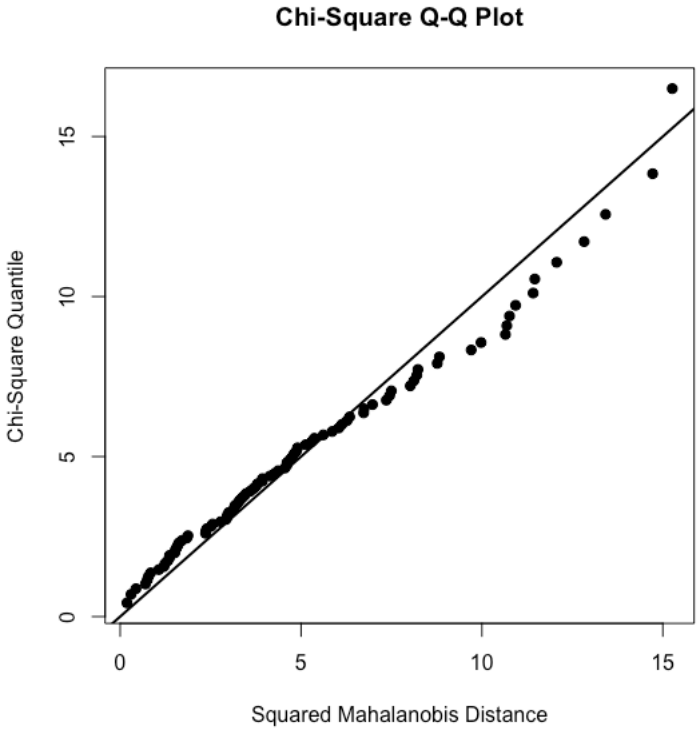


Figure 4. Multivariate Q-Q plot for normality of log transformed data.

Table 6. Multivariate tests for skewness and kurtosis for log transformed data.

Test	Statistic	P Value
Mardia Skewness	53.595	0.023*
Mardia Kurtosis	1.564	0.117

Table 7. Univariate Tests for normality of log transformed dependent variables.

Protein	Shapiro-Wilks	P value
KEAP1	0.9800	0.180
Cyt. Nrf2	0.9733	0.0606
Nuc. Nrf2	0.9808	0.2086
SOD1	0.9802	0.1857
HO1	0.9880	0.5832

Table 8. Univariate normality measures for log transformed dependent variables. Mean and standard deviation values are from log-transformed, normalized to GAPDH. Acceptable skew values were expected to be within -2 to +2; kurtosis values expected in the range of -7 to +7.

Protein	N	Mean	Std.Dev.	Skew	Kurtosis
KEAP1	92	-0.41251	0.60298	-0.05269	0.97200
Cyt. Nrf2	92	0.66820	0.55935	0.04232	0.98431
Nuc. Nrf2	92	-0.42676	0.52828	-0.19063	0.71533
SOD1	92	-0.93137	0.48827	0.27948	-0.06532
HO1	92	-0.03874	0.32670	-0.12506	0.82222

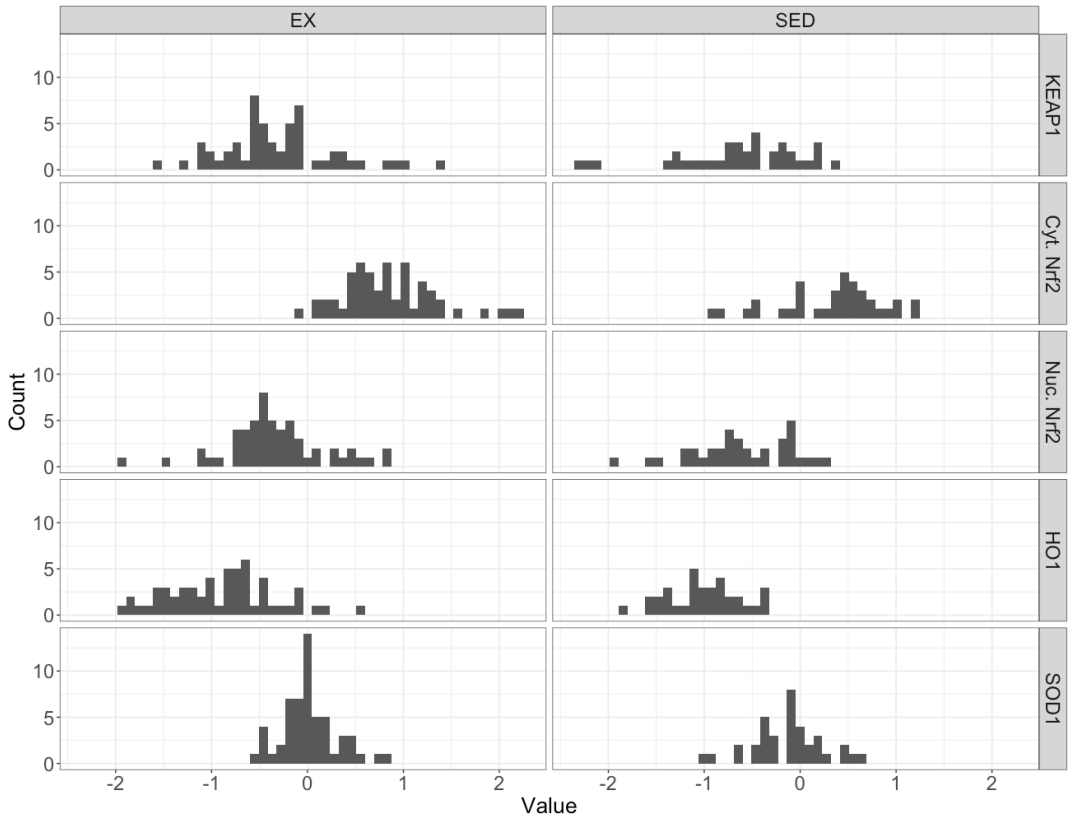


Figure 5. Histograms of protein levels, normalized to GAPDH, separated by exercise/sedentary group for log transformed data.

Interaction Effect

Table 9. Results from the test for interaction effect between muscle type and exercise on protein using a repeated measures multivariate analysis of variance.

Statistic	Value	F Value	Numerator Degrees of Freedom	Denominator degrees of freedom	P value
Wilks' Lambda	0.423	0.636	15	7	0.782
Pillai's Trace	0.577	0.636	15	7	0.782
Hotelling-Lawley Trace	1.362	0.636	15	7	0.782
Roy's Greatest Root	1.362	0.636	15	7	0.782

The test for interaction effects between exercise and muscle type revealed no significant effect (Wilks' = 0.432, F = 0.636, p = .782). Main effects were examined as a follow-up.

Exercise Effect

A repeated-measures multivariate analysis of variance showed no overall difference in proteins between exercise and sedentary groups (Wilks = .610, F = 2.171, p = .106).

Table 10. Results from the test for effect of exercise on protein using a repeated measures multivariate analysis of variance.

Statistic	Value	F Value	Numerator Degrees of Freedom	Denominator degrees of freedom	P value
Wilks' Lambda	0.610	2.171	15	17	0.106
Pillai's Trace	0.390	2.171	15	17	0.106
Hotelling-Lawley Trace	0.639	2.171	15	17	0.106
Roy's Greatest Root	0.639	2.171	15	17	0.106

Muscle Effect

Table 11. Results from the test for effect of muscle on protein using a repeated measures multivariate analysis of variance.

Statistic	Value	F Value	Numerator Degrees of Freedom	Denominator degrees of freedom	P value
Wilks' Lambda	0.192	15.44	15	7	0.186
Pillai's Trace	0.808	15.44	15	7	0.186
Hotelling-Lawley Trace	4.213	15.44	15	7	0.186
Roy's Greatest Root	4.213	15.44	15	7	0.186

There was no main effect of muscle type on protein levels (Wilks' = .192, F = 15.44, p = 0.186). This suggests that at the multivariate level, muscles do not differ in their protein levels between exercise and sedentary groups.

Univariate Analyses

Given that multivariate normality violations occurred even after transformation and observed power was low (0.439) due to low sample size and skewness violations, univariate repeated measures ANOVAs were performed. Results from each univariate analysis are listed individually below.

*Table 12. Univariate ANOVA table for log transformed cytosolic KEAP1. *denotes significance at the alpha = 0.05 level. Sphericity was violated, and within-subjects results are reported as Greenhouse-Geisser test results.*

Protein	Error	Measure	Df	Type III Sum of Sq.	Mean Sq.	F value	P
KEAP1	Between	Exercise	1	2.512	2.512	2.90	0.104
		Residuals	21	18.220	0.868		
	Within	Muscle	2.122	0.965	0.455	1.450	0.245
		Muscle x Exercise	2.122	0.473	0.223	0.711	0.505
		Residuals	63	13.980	0.222		

There was no significant effect of exercise, muscle, or interaction terms on KEAP1 concentration. Due to the low power of this study and a trend towards significance, the results do not necessarily suggest that there is no effect of exercise on KEAP1. Rather we cannot suggest that there is a statistically significant effect using this methodology and sample size.

*Table 13. Univariate ANOVA table for log transformed cytosolic Nrf2. *denotes significance at the alpha = 0.05 level.*

Protein	Error	Measure	Df	Type III Sum of Sq.	Mean Sq.	F value	P
Cytosolic Nrf2	Between	Exercise	1	1.5522	1.5522	2.768	0.111
		Residuals	21	11.776	0.5608		
	Within	Muscle	3	0.192	0.064	0.340	0.797
		Muscle x Exercise	3	0.974	0.325	1.728	0.170
		Residuals	63	11.845	0.188		

Similar to KEAP1, there is an apparent non-significant effect of exercise on Nrf2 that could be attributed to low power and could provide significance if a larger sample size or different methodology was utilized. However, lower F values for muscle and

interaction terms (0.340 and 1.728, respectively) suggest that there is no significant trend in these terms.

*Table 14. Univariate ANOVA table for log transformed nuclear Nrf2. *denotes significance at the alpha = 0.05 level.*

Protein	Error	Measure	Df	Type III Sum of Sq.	Mean Sq.	F value	P
Nuclear Nrf2	Between	Exercise	1	5.695	5.695	8.977	0.007*
		Residuals	21	13.324	0.634		
	Within	Muscle	3	0.535	0.178	1.317	0.277
		Muscle x Exercise	3	1.161	0.387	2.858	0.044*
		Residuals	63	8.528	0.135		

Nuclear Nrf2 noted a significant interaction effect (F = 2.858, p = 0.044), which may cause issues when interpreting the main effects, so both simple main effects (Table 14) and pairwise comparisons were made using Tukey post hoc corrections (Table 15).

Table 15. Simple main effects for the nuclear Nrf2 repeated measures ANOVA.

Protein	Muscle	SS	Df	Mean Sq.	F value	P
Nuclear Nrf2	Gastrocnemius	1.292	1	5.695	4.438	0.047*
	Soleus	1.041	1	0.634	6.082	0.022*
	Superficial Quad	0.320	1	0.178	1.079	0.205
	Deep Quad	4.203	1	0.387	10.756	0.004*

Simple main effects illustrated significant differences between exercise groups in the gastrocnemius (F = 4.438, p = 0.047), soleus (F = 6.082, p = 0.022), and deep quadriceps (F = 10.756, p = 0.004). This suggests that exercise increases nuclear-located Nrf2 in gastrocnemius, soleus, and deep quadriceps muscle, but not superficial quadriceps.

Table 16. Univariate ANOVA table for log transformed cytosolic HO1. *denotes significance at the alpha = 0.05 level.

Protein	Error	Measure	Df	Type III Sum of Sq.	Mean Sq.	F value	P
HO1	Between	Exercise	1	0.27	0.2700	0.392	0.538
		Residuals	21	13.76	0.6882		
	Within	Muscle	3	1.883	0.628	8.157	<0.001*
		Muscle x Exercise	3	0.221	0.074	0.956	0.419
		Residuals	63	4.848	0.077		

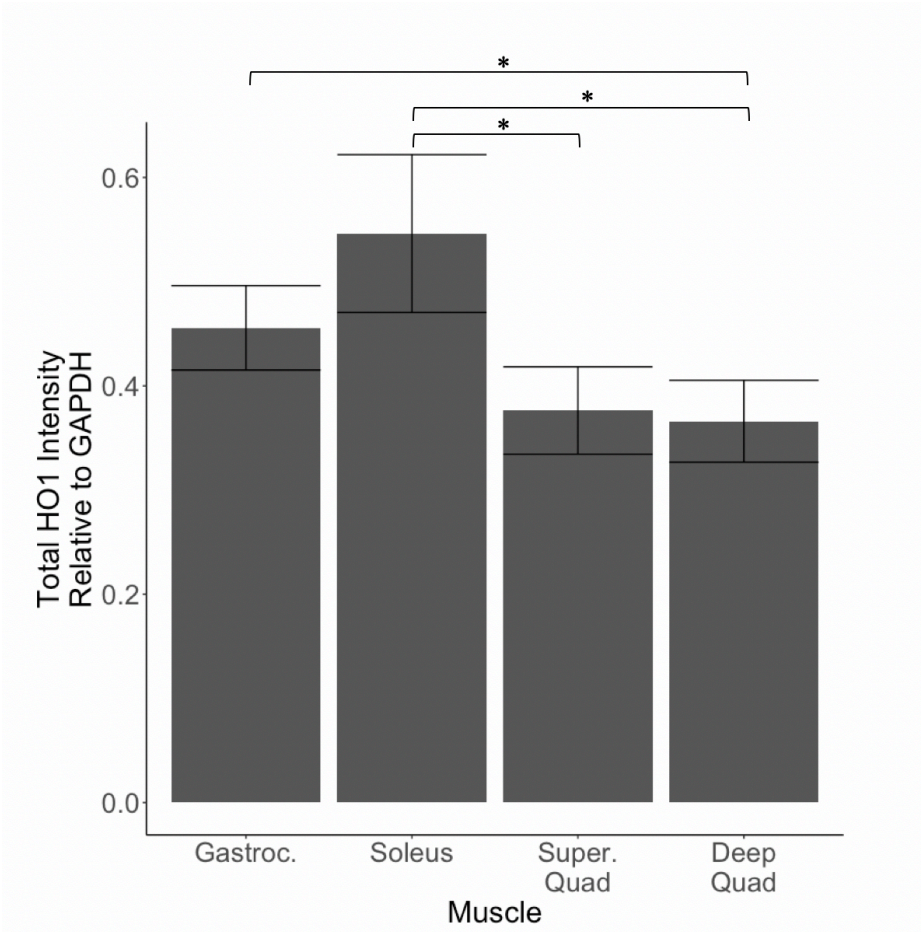


*Figure 6. Total HO1 response by muscle. Data represented is untransformed, raw data. *denotes significant differences at p < 0.05 between groups determined by Bonferroni adjustment.*

Heme oxygenase-1 did not show a significant interaction effect nor a main effect of exercise. However, it did show a significant difference across muscles ($F = 8.157$, $p < 0.001$).

This indicates that muscles differ in overall quantity of HO1 but does not appear to change with significantly with the exercise in this study. In this case, the gastrocnemius was significantly higher than from the deep quadriceps muscle, and the soleus was significantly greater than both superficial and deep quadriceps in HO1 quantity.

*Table 17. Univariate ANOVA table for log transformed cytosolic SOD1. *denotes significance at the alpha = 0.05 level.*

Protein	Error	Measure	Df	Type III Sum of Sq.	Mean Sq.	F value	P
SOD1	Between	Exercise	1	0.4306	0.4306	3.887	0.062
		Residuals	21	2.3262	0.1108		
	Within	Muscle	3	1.979	0.660	9.379	<0.001*
		Muscle x Exercise	3	0.467	0.156	2.215	0.095
		Residuals	63	4.432	0.070		

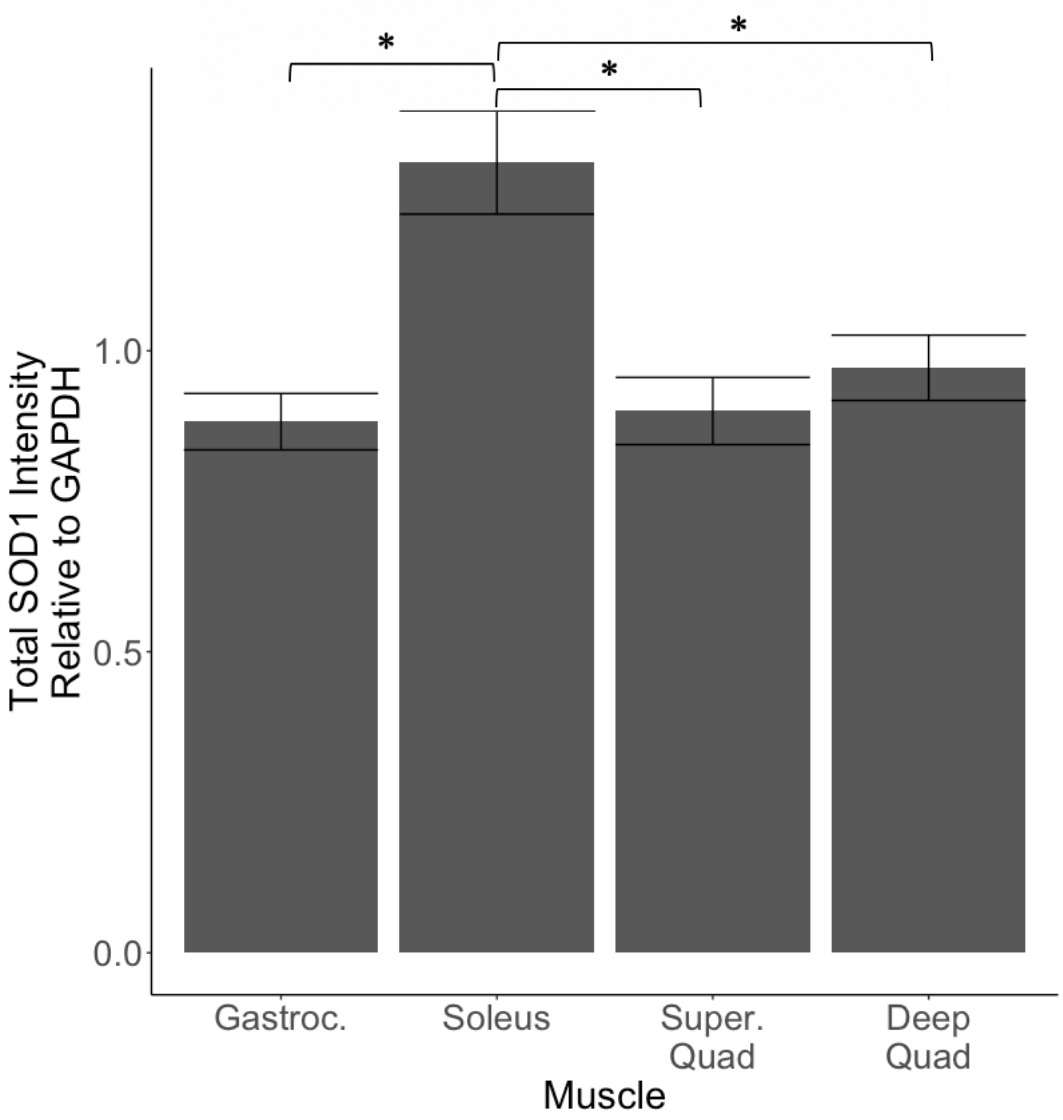


*Figure 7. Total SOD1 response by muscle. Data represented is untransformed, raw data. *denotes significant differences at p < 0.05 between groups determined by Bonferroni adjustment.*

Superoxide dismutase 1's p-value similarly did not reach the *a priori* alpha level of 0.05 for the exercise main effect ($F = 3.887$, $p = 0.062$), but did have a muscle significant muscle effect ($F = 9.379$, $p < 0.001$). This means the exercise protocol used in this study did not reach statistical significance to induce a significant change in SOD1

levels in all muscles, but with a p-value of 0.062, it is worth considering that methodological changes, namely sample size, would likely bring it into the range of significance. However, the differences in muscles remains highly significant, reflecting a difference in SOD1 concentrations between muscles. The soleus demonstrated increased SOD1 relative to all other muscle groups, with no other noted differences.

It is also worth noting that the exercise by muscle interaction effect was not statistically significant but did have a p-value less than 0.1, indicating consideration for future research. This would indicate that all muscles examined did not have similar changes in SOD1 in response to this particular exercise training, so this makes interpretation of the significance more difficult.

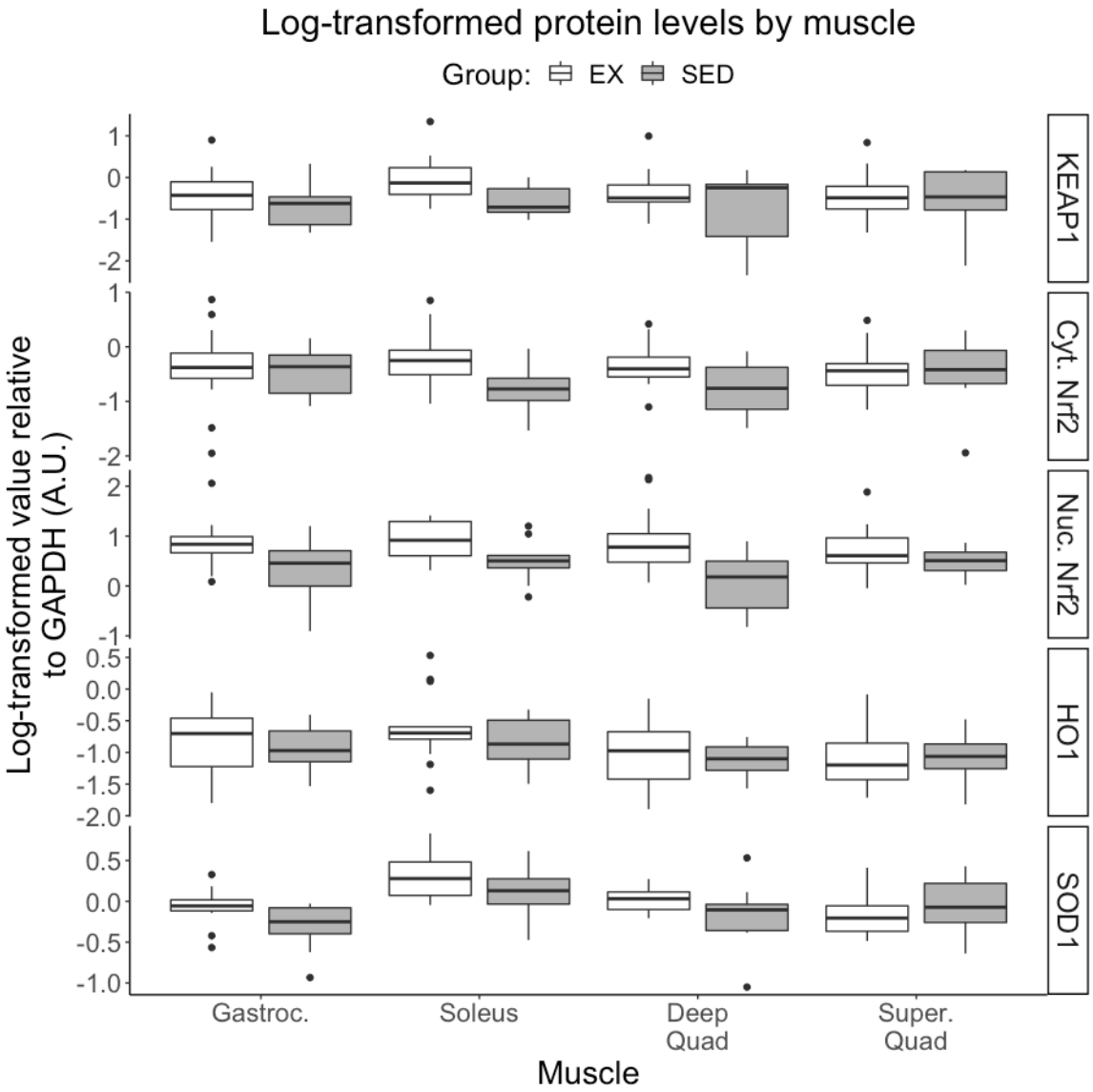


*Figure 8. Log-transformed protein levels in different muscles for both sedentary and exercise groups. The data is represented as boxplots where the box is the inter-quartile range(Q1 to Q3), whiskers represent the most extreme value in the theoretical range of data (Q1 – 1.5*IQR; Q3+ 1.5*IQR), and dots represent outliers outside of the theoretical range of data.*

Figure 8 demonstrates visual differences in all proteins observed in the study, separated by muscle and exercise group for visual purposes. This graph provides a visual summary of the data, without any statistical measures or differences, for clarity purposes.

KEAP1/Nrf2 Ratio

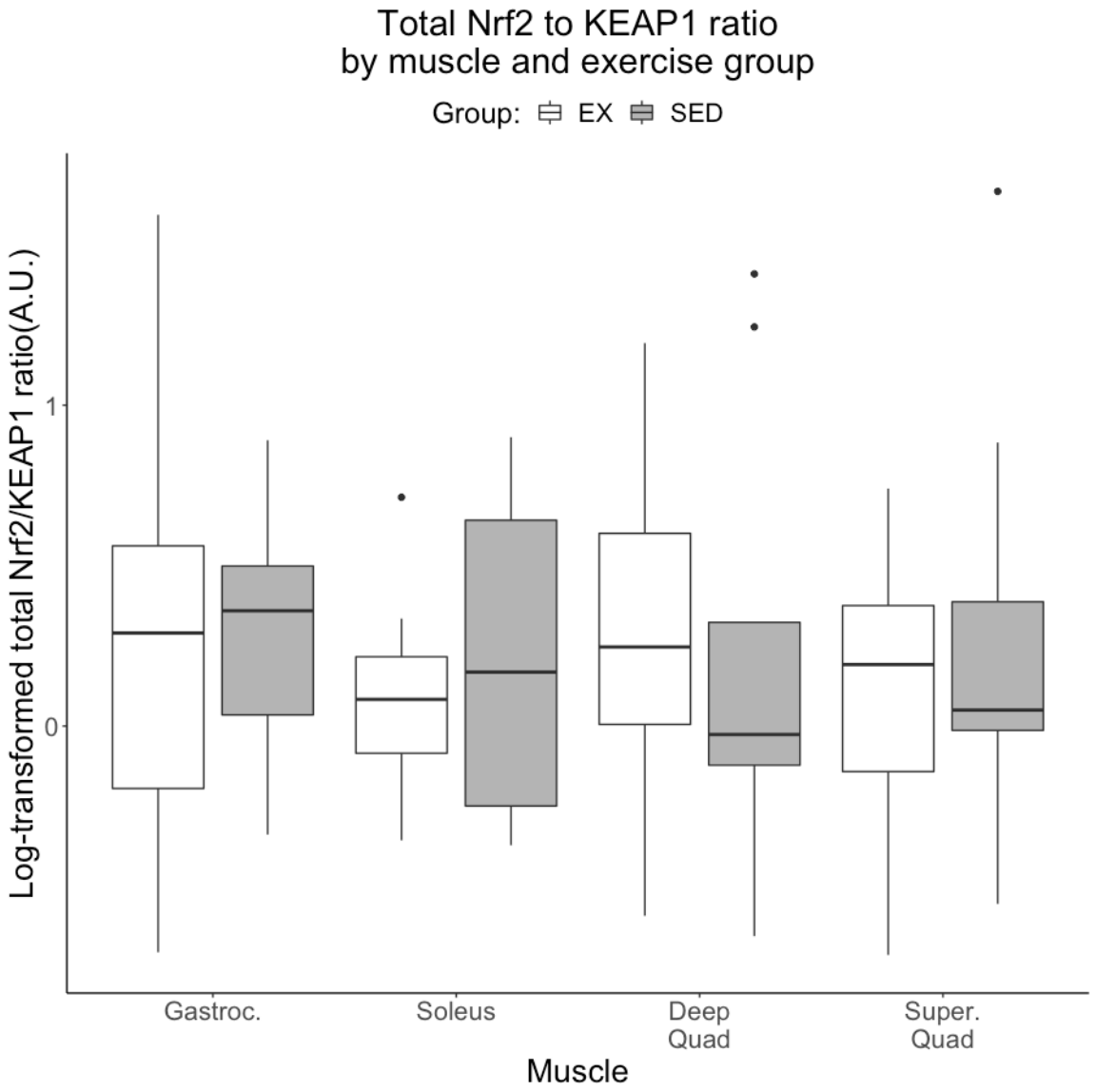


*Figure 9. Log-transformed /KEAP1 expressed as a ratio in each muscle between exercise and sedentary groups. The data is represented as boxplots where the box is the inter-quartile range(Q1 to Q3), whiskers represent the most extreme value in the theoretical range of data (Q1 – 1.5*IQR; Q3+ 1.5*IQR), and dots represent outliers outside of the theoretical range of data.*

*Table 18. Univariate ANOVA table of log transformed Nrf2/KEAP1 ratio. *denotes significance at the alpha = 0.05 level.*

	Error	Measure	Df	Type III Sum of Sq.	Mean Sq.	F value	P
Total Nrf2/KEAP1 ratio	Between	Exercise	1	0.101	0.1008	0.205	0.655
		Residuals	21	10.317	0.4913		
	Within	Muscle	3	0.293	0.09753	0.562	0.642
		Muscle x Exercise	3	0.094	0.03140	0.181	0.909
		Residuals	63	10.932	0.17353		

The total ratio of Nrf2/KEAP1, log transformed, was not significant for exercise, muscle, or muscle by exercise interaction effects using a repeated-measures ANOVA. This suggests that the ratio of these two does not change between exercise or muscle.

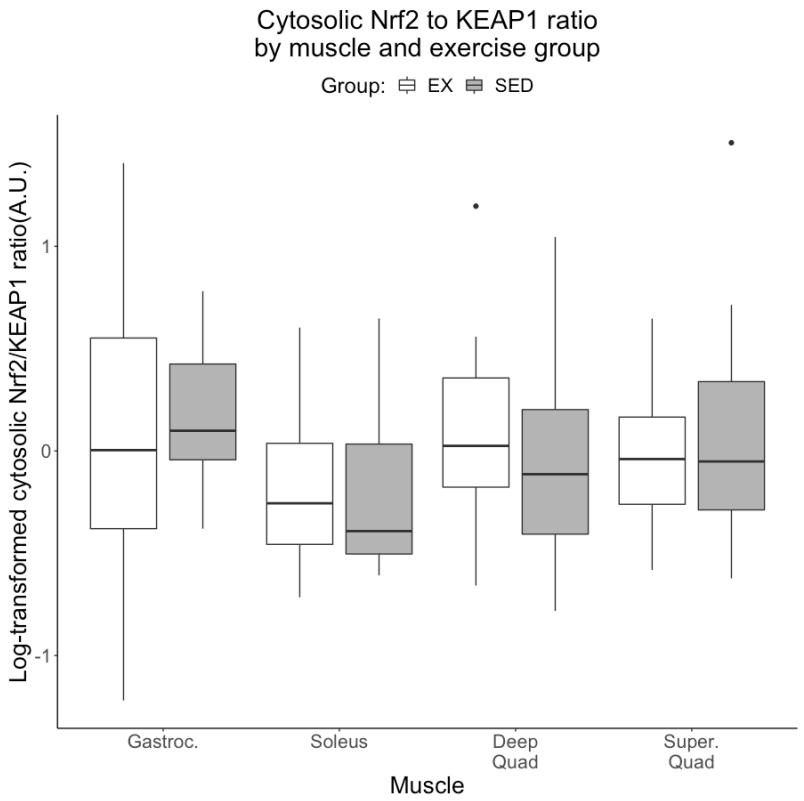


*Figure 10. Log transformed cytosolic Nrf2/KEAP1 ratios in each muscle by group. The data is represented as boxplots where the box is the inter-quartile range(Q1 to Q3), whiskers represent the most extreme value in the theoretical range of data (Q1 – 1.5*IQR; Q3+ 1.5*IQR), and dots represent outliers outside of the theoretical range of data.*

Table 19. Univariate ANOVA table for log transformed Cytosolic ratio of Nrf2/KEAP1. *denotes significance at the alpha = 0.05 level.

	Error	Measure	Df	Type III Sum of Sq.	Mean Sq.	F value	P
Cytosolic Nrf2/KEAP1 ratio	Between	Exercise	1	0.155	0.155	0.278	0.604
		Residuals	21	8.695	0.414		
	Within	Muscle	3	0.983	0.3277	1.498	0.224
		Muscle x Exercise	3	0.131	0.0436	0.199	0.897
		Residuals	63	13.777	0.2187		

A univariate repeated measures ANOVA was performed for Nrf2/KEAP1 ratio for total amounts (nuclear + cytosolic), as well as cytosolic only, in order to assess

whether or not the ratio changed due to exercise. Results demonstrate no significant effect of exercise on the Nrf2/KEAP1 ratio, either expressed as total Nrf2/KEAP1 ($F = 0.467$, $p = .585$) or as cytosolic Nrf2/KEAP1 ($F = .379$, $p = .545$).

Correlations between Nrf2 and Antioxidant Production

In order to better understand if inclusion of KEAP1, in the form of the Nrf2/KEAP1 ratio, Pearson correlations were conducted to examine the strength of relationship increased between signaling molecules and down-stream antioxidants. Nuclear-located Nrf2 was compared with HO1 and SOD1 as the 'baseline' for strength of relationship between signaling molecule and antioxidant production (Figures 11, 13). The relationships were also divided by exercise and sedentary groups to determine if the relationships were any different between groups (Figures 12, 14). These analyses were then conducted with Nrf2/KEAP1 ratios to assess whether or not the relationship strengthened. Both cytosolic Nrf2/KEAP1 (Figures 15-18) and total Nrf2/KEAP1 (Figures 19-22) ratios were used since there is no current consensus on how the ratio should be measured.

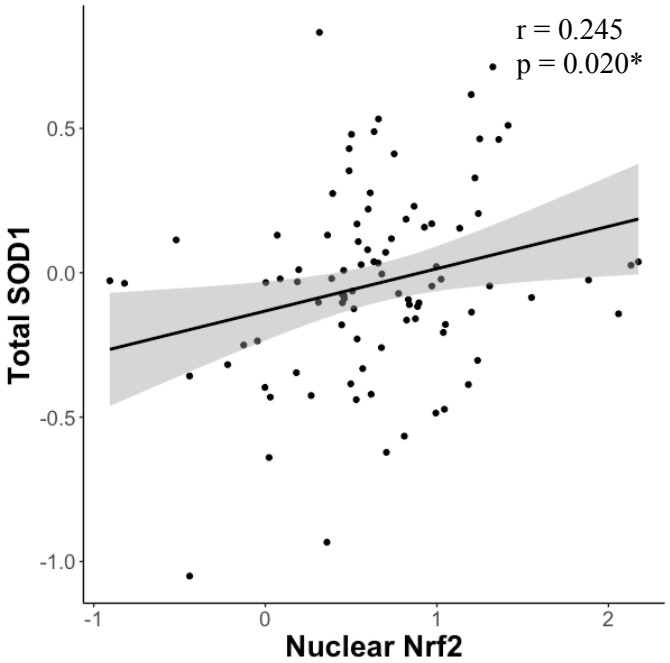


Figure 11. Relationship between nuclear Nrf2 and total SOD1 content (both variables log transformed).

Nuclear Nrf2 significantly correlated with corresponding Total SOD1 measures in mice, regardless of exercise group and muscle (Figure 9). When broken up by exercise group, neither correlation was significant (Figure 10) but approaching significance in the sedentary group.

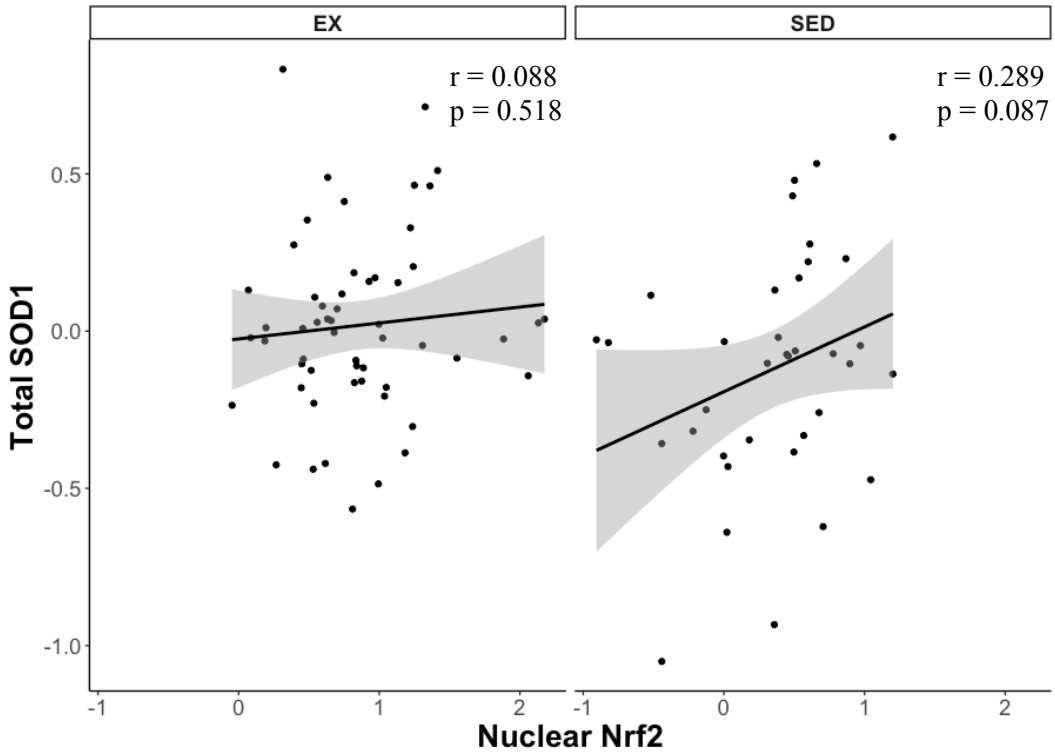


Figure 12. Relationship between Nrf2 and total cell SOD1 in each group (both variables log transformed).

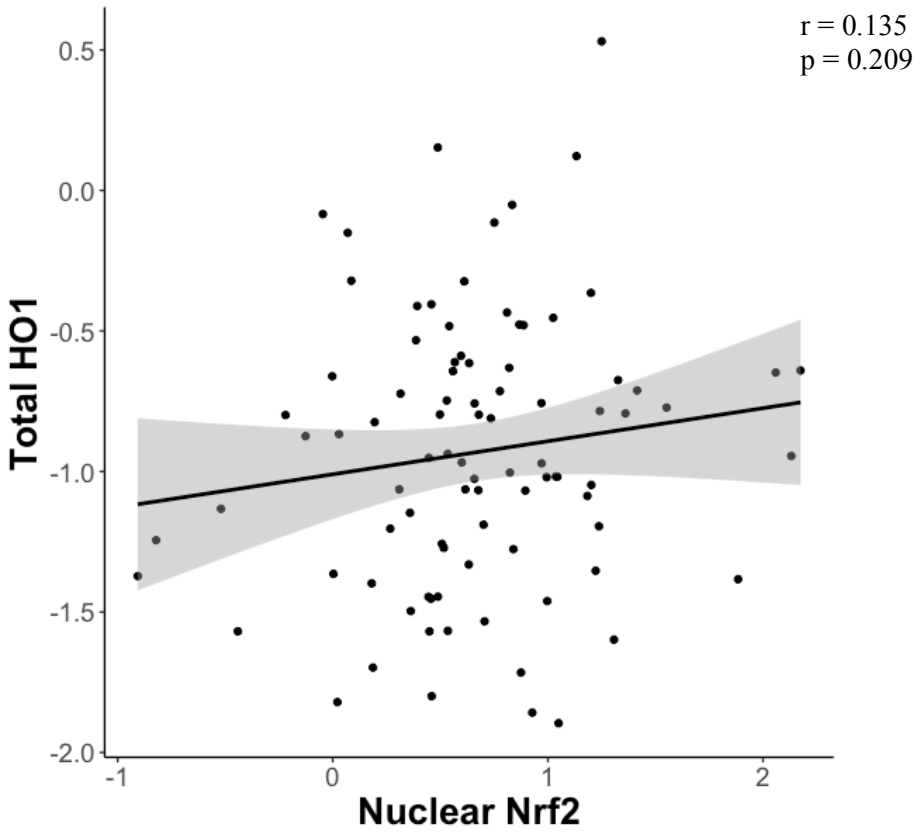


Figure 13. Relationship between nuclear Nrf2 and total HO1 content (both variables log transformed).

Nuclear Nrf2 did not significantly correlate with total HO1 when examining across all muscle groups and exercise groups (Figure 11). However, when split into exercise groups, the sedentary group had a significant correlation (Figure 12), whereas the exercise group did not.

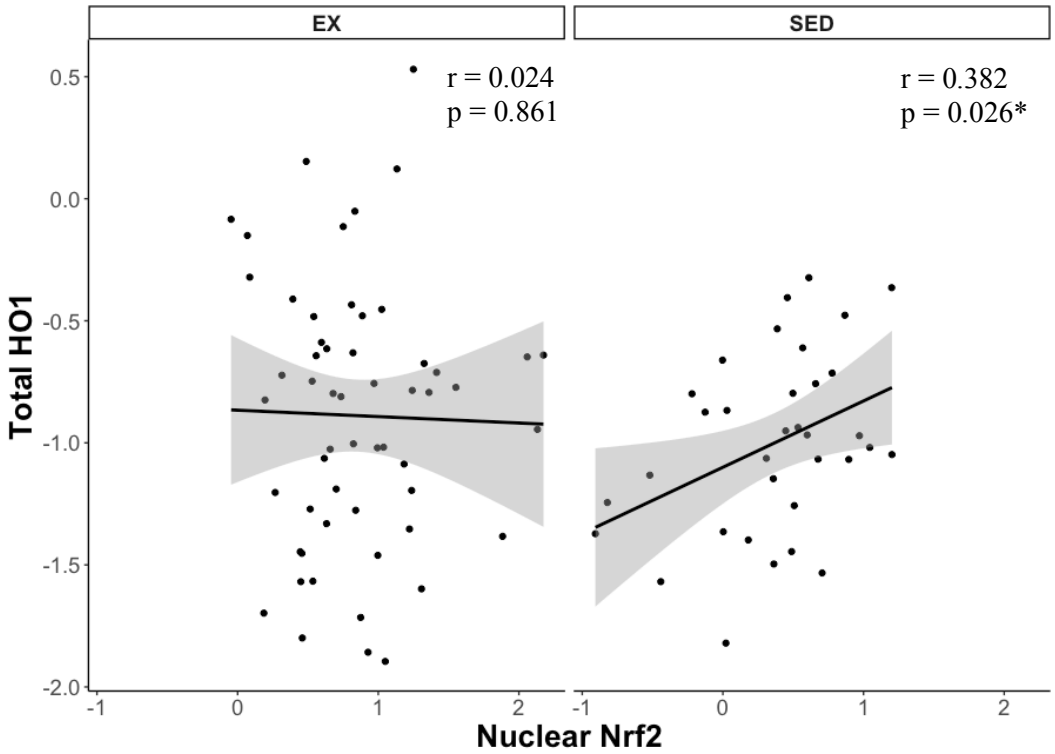


Figure 14. Relationship between nuclear Nrf2 and total cell HO1 in each group (both variables log transformed).

Correlations Between Nrf2/KEAP1 Ratios and Antioxidants

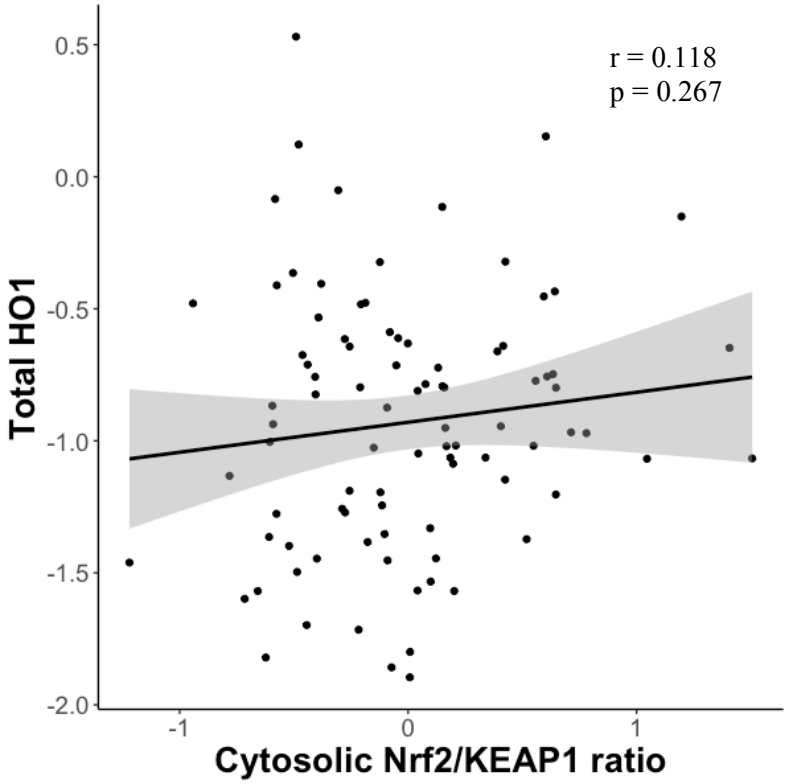


Figure 15. Relationship between cytosolic Nrf2/KEAP1 ratio and total HO1 content (both variables log transformed).

The cytosolic Nrf2/KEAP1 ratio did not appear to correlate with total HO1 when examined with all muscles and exercise groups together (Figure 13), nor when examined as separate groups based on exercise (Figure 14).

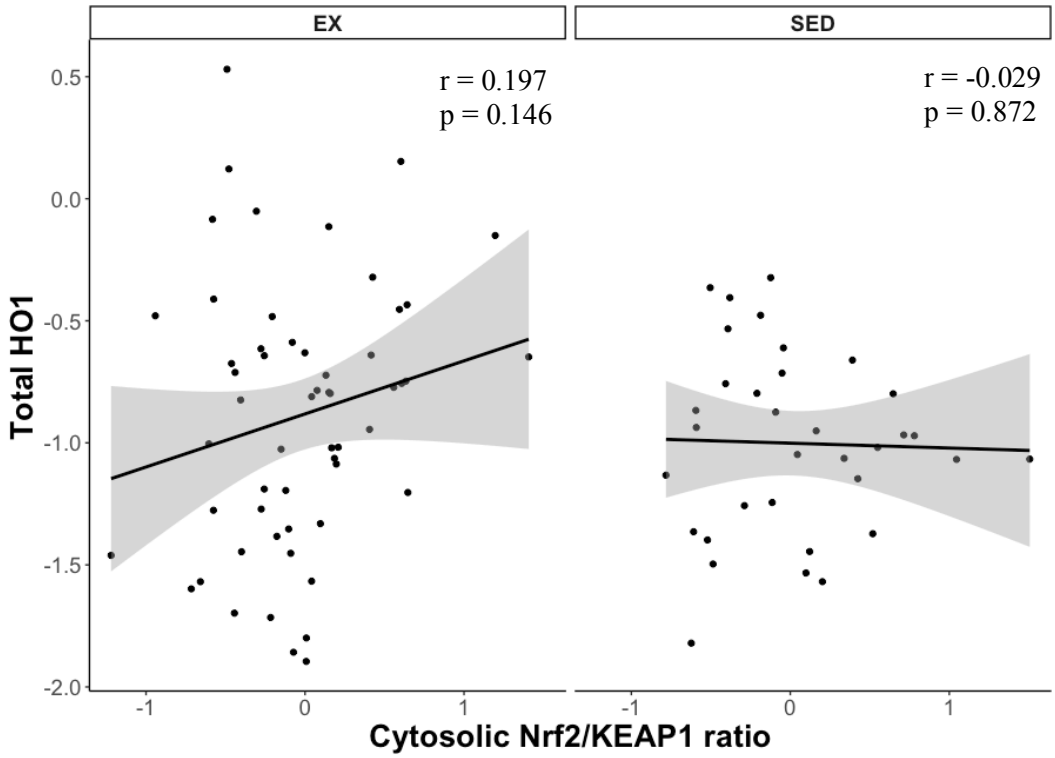


Figure 16. Relationship between cytosolic Nrf2/KEAP1 ratio and total cell HO1 in each group (both variables log transformed).

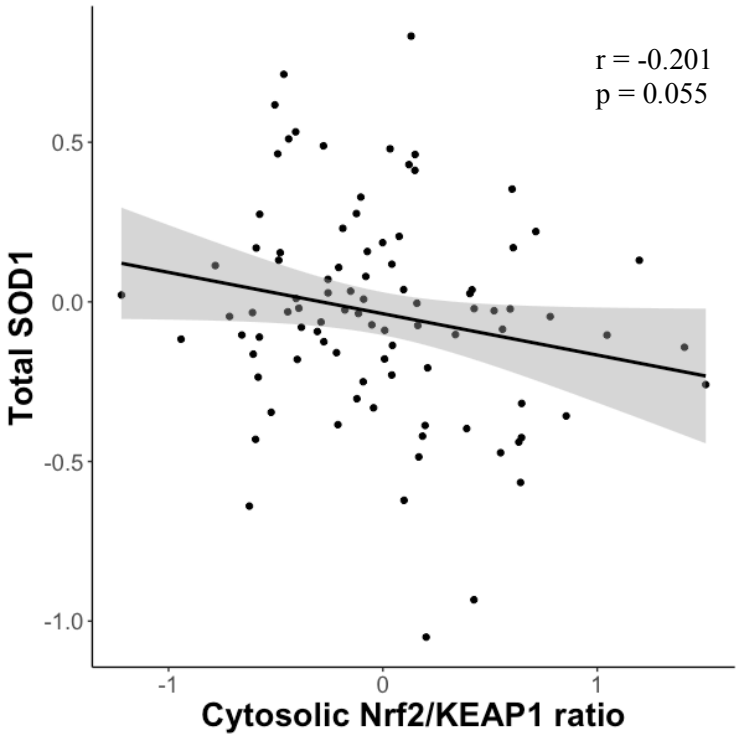


Figure 17. Relationship between cytosolic Nrf2/KEAP1 ratio and total cell SOD1 (both variables log transformed).

Cytosolic Nrf2 to KEAP1 ratio was near significantly correlated with Total SOD1 (Figure 15). Of particular note, this was a negative correlation, meaning as the relative

amounts of Nrf2 to KEAP1 decreased, total SOD1 decreased. When divided by exercise groups, the correlations remained negative, but was not near significance.

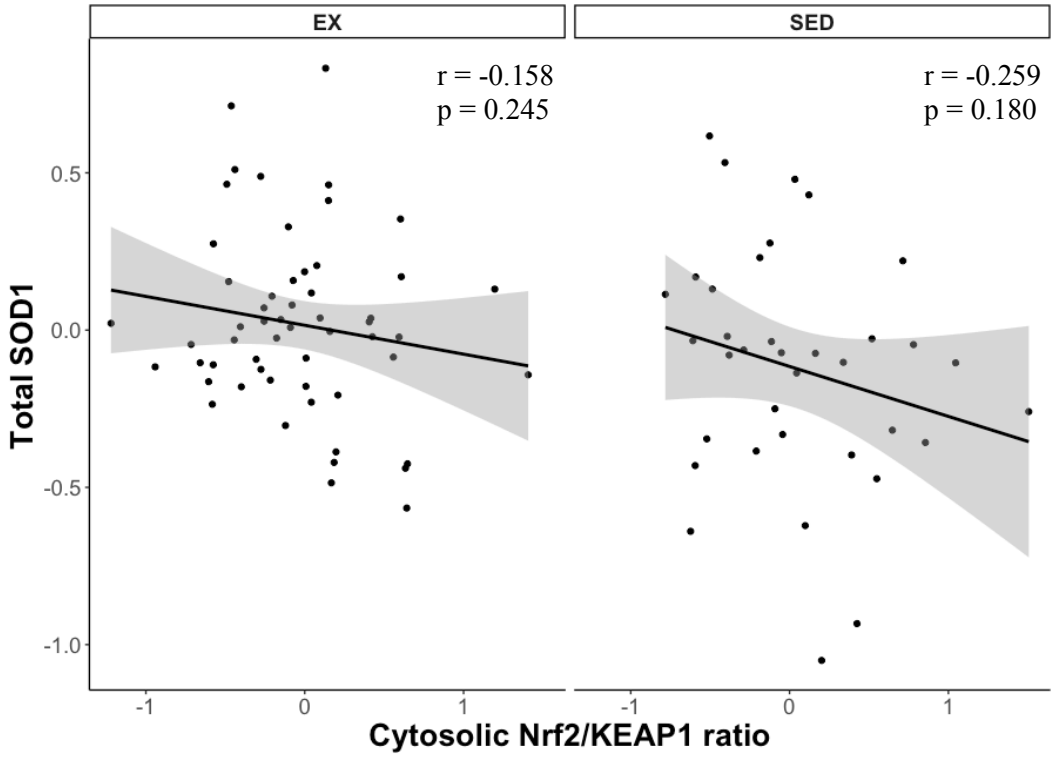


Figure 18. Relationship between cytosolic Nrf2/KEAP1 ratio and total cell SOD1 in each group (both variables log transformed).

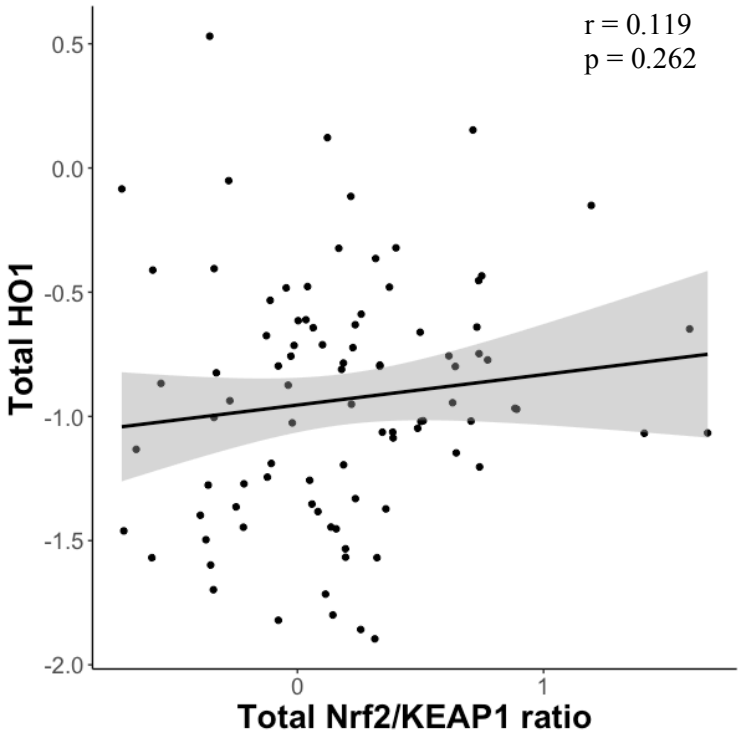


Figure 19. Relationship between total cell Nrf2/KEAP1 ratio and total cell HO1 (both variables log transformed).

Total Nrf2/KEAP1 ratio was not significantly correlated with total HO1 across muscles and exercise groups (Figure 17). When divided by exercise group, correlations remained insignificant (Figure 18).

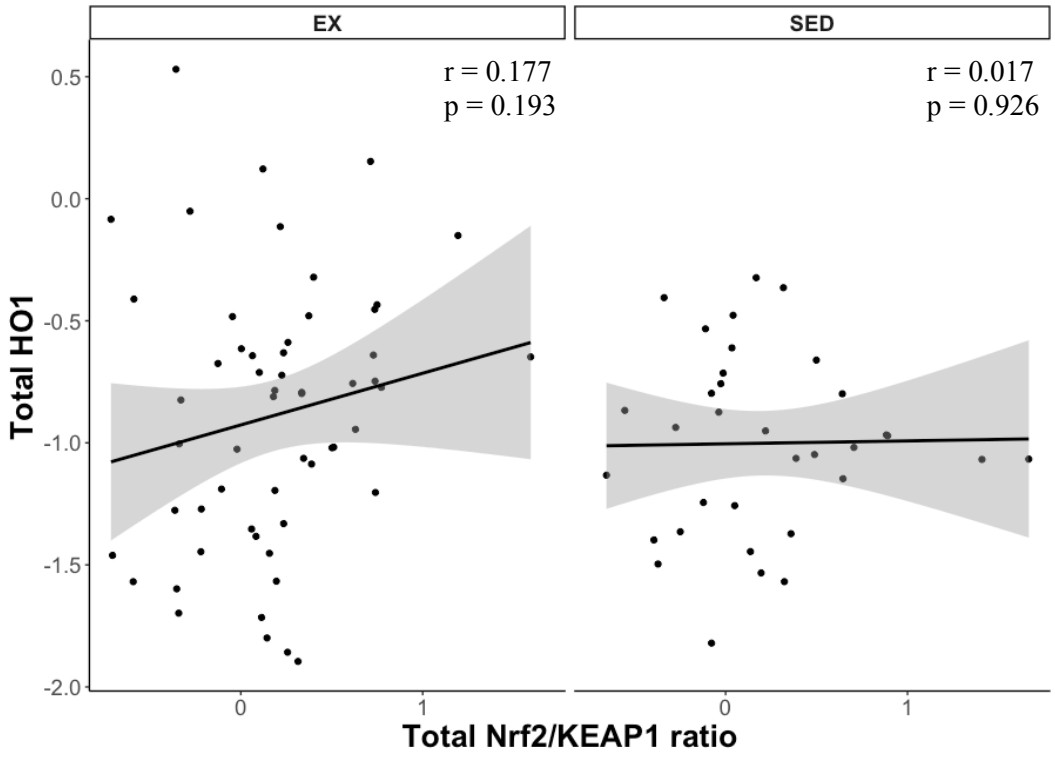


Figure 20. Relationship between total cell Nrf2/KEAP1 ratio and total cell HO1 in each group (both variables log transformed).

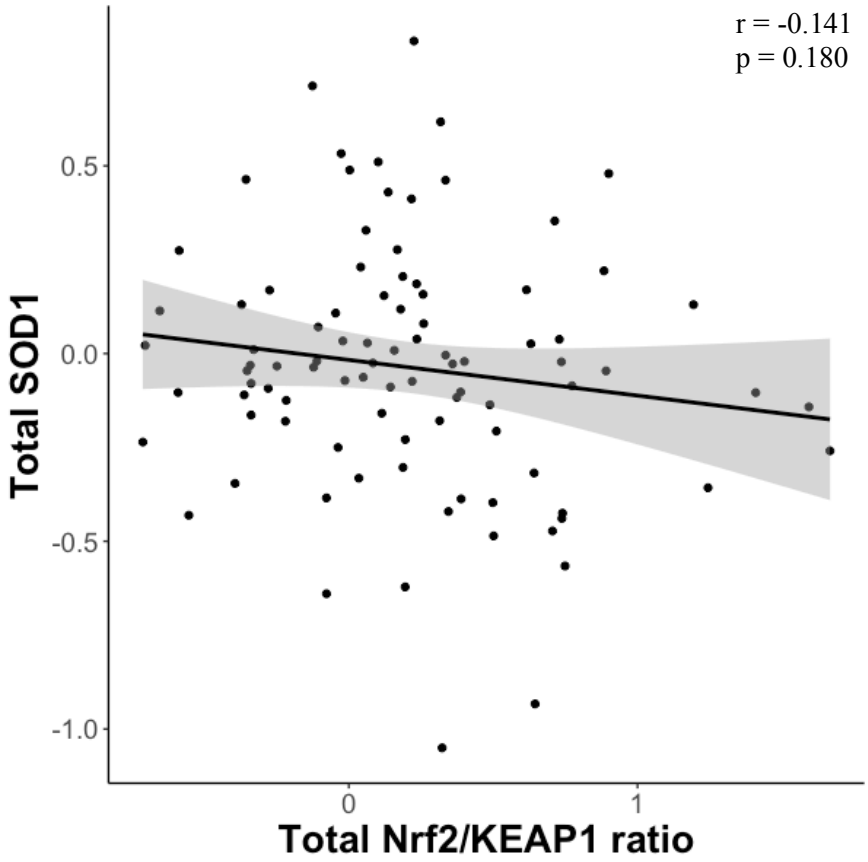


Figure 21. Relationship between total cell Nrf2/KEAP1 ratio and total cell SOD1 (both variables log transformed).

Total Nrf2/KEAP1 ratio was not significantly correlated with total SOD1 across muscles and exercise groups (Figure 19). When divided by exercise group, correlations between total Nrf2/KEAP1 and total SOD1 also were not statistically significant (Figure 20).

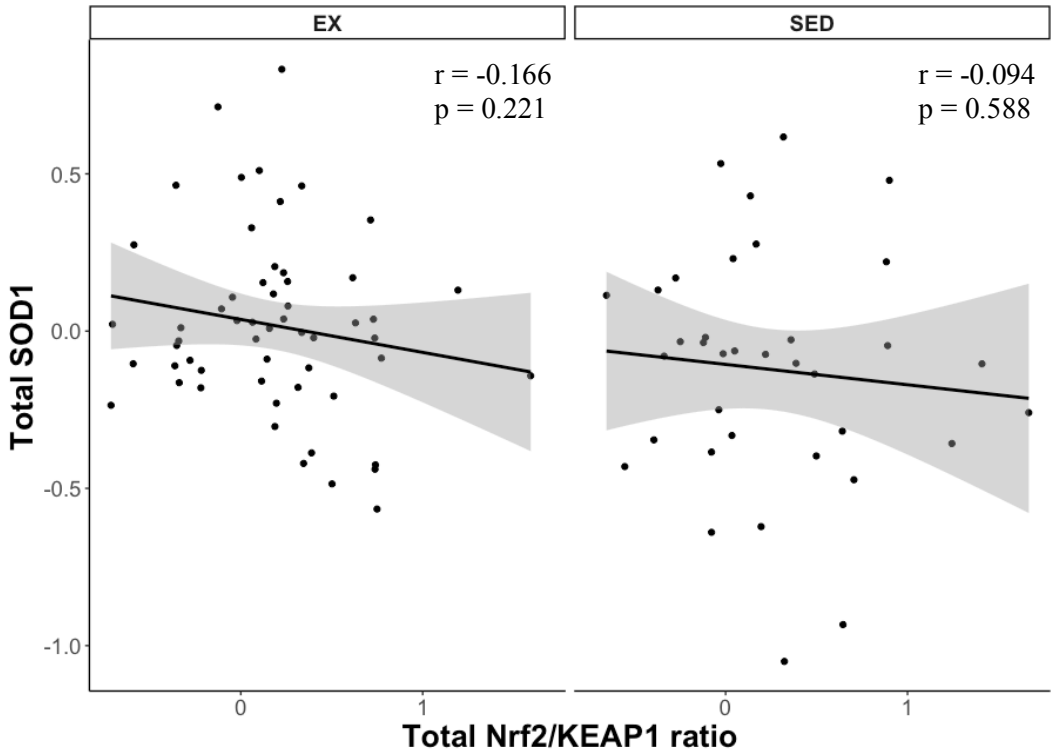


Figure 22. Relationship between total cell Nrf2/KEAP1 ratio and total cell SOD1 in each group (both variables log transformed).

CHAPTER V

DISCUSSION

Multivariate Analysis

When tests for multivariate normality were conducted, the original data demonstrated violations in Mardia tests for skewness and kurtosis (Table 4). Additionally, Shapiro-Wilks tests for univariate normality showed violations in every dependent variable (Table 5). Data were transformed using natural log transformations in order to transform the data into a normal distribution. After transformation, multivariate kurtosis was no longer violated, but multivariate skewness was still violated. Univariate analysis of log-transformed dependent variables showed no violation of normality, using the Shapiro-Wilks test, nor showing high skew or kurtosis.

As a result of the transformations and normality, the multivariate rm-ANOVA was still performed to assess performance of the model. For the main effect of exercise, there was no significant effect (Wilks' Lambda = 0.610, F = 2.171, df = 15/7, p = 0.106). Additionally, there was no significant effect within subjects of muscle (Wilks' Lambda = 0.192, F = 15.44, df = 15/7, p = 0.186). Lastly, the interaction effect between exercise and muscle was not significant (Wilks' Lambda = 0.423, F = 0.636, df = 15/7, p = 0.782). It is important to note that observed power from the rm-MANOVA for main effect of exercise was 0.439; therefore, it is highly possible that the low power was responsible for

lack of significant differences between exercise and sedentary groups. Considering power is equal to 1 minus beta, beta equals 0.561, indicating a 56.1% chance of a type II error.

Considering the assumptions of multivariate ANOVAs, tests were performed to assess each of the assumptions and which ones may have been violated. The results of these tests revealed that there are in fact multivariate outliers and violations in skewness. While some suggest that rm-MANOVAs are robust to violations in skewness[58], high kurtosis is suggested to decrease power substantially in a multivariate test[59]. Low power could, however, indicate that the overall effect size is small and may ultimately be a 'true' negative finding. Any future replication of this study should, at minimum, include a larger sample size in an attempt to increase power. However, given these findings and considering low sample size in addition to no violations in univariate findings, univariate tests were conducted to explore the relationship between exercise and muscles for each protein more clearly.

KEAP1/Nrf2 Pathway

KEAP1 appeared to have no statistically significant trend in exercise, muscle, or interaction. While not statistically significant, it is worth noting that means tended to be slightly elevated in exercise groups in all but the superficial quad, suggesting that increasing sample size or training effect may provide the ability to detect a significant difference in some muscles. While future studies addressing increased effect and/or power are suggested, these are some of the first findings to represent changes in KEAP1 with regular exercise training.

Cytosolic Nrf2 also did not show any significant changes across exercise, muscle, or any interaction of the two. While this may conflict with some current findings that *whole cell* Nrf2 increases with exercise training[23,23], it is worth noting that non-significance may be confounded by the gastrocnemius and superficial quadriceps. Many findings to date have been done in myocardial tissue, which is highly aerobic[60], and has not been confounded by more glycolytic-based muscle types. The more oxidative tissues, soleus and deep quadriceps, both illustrated higher differences in means between exercise and sedentary groups. This is more to be expected, since oxidative stress has been shown to occur at higher rates during exercise and antioxidant pathways are more active in these tissues[1].

Nuclear Nrf2 levels, however, did show a significant exercise interaction effect. Because of this, the main effects could be misleading, and as such, pairwise-comparisons were made in order to determine which pairs of exercise and muscle were different from one another. What was interesting in these findings is that only the deep quad in the sedentary group was significantly different from almost all other exercise groups. That is to say that it had the least amount of nuclear Nrf2, whereas the exercised tissue was typically the highest in average values. The 'main effect' finding that was significant in the repeated measures ANOVA was thus largely due to this particular group and warrants further investigation. Based on the understanding of oxidative vs glycolytic muscle fibers, there was an expectation that deep quadriceps and soleus would exhibit similar responses in nuclear Nrf2. However, our data suggest that they were not similar, as soleus muscles did not significantly differ from other muscle group-exercise combinations.

While some of this is due to reduced power of the study, this may require further investigation to figure out why this phenomenon occurred.

One possible explanation is muscle usage during exercise. While dynamics of muscle recruitment during exercise in mice may be hard to determine, it is suggested that there is a difference in forces applied by each muscle, where soleus provides more negative horizontal forces and quadriceps provide more positive horizontal forces[61], indicating a difference between contraction type (eccentric vs. concentric) and thus, metabolic load. Additionally, citrate synthase changes incurred by exercise indicate that red vastus has a higher relative change after training compared to the relative change in the soleus, indicating potentially higher metabolic demands[62]. As such, although phenotypically similar as 'oxidative fibers', the overall usage and recruitment during treadmill running may indicate that there could be differences even among fiber types.

While the observed translocation of Nrf2 in the deep quadriceps supports many of the models that exist around activation of Nrf2, via translocation to the nucleus, many of the studies making these suggestions have not measured protein levels of Nrf2 in the nucleus in comparison to cytosolic Nrf2. Of those that have examined nuclear Nrf2, these results are congruent with their findings of increased nuclear Nrf2 after regular exercise training. Asghar et al. reported increases in nuclear Nrf2 in renal tissue from rats[49]; George et al. found an increase in nuclear Nrf2 only in older rats in renal tissue, but no change in younger rats[51]; Gounder et al. found increases in nuclear Nrf2 in myocardium of both young and old exercised mice[17]; Sun et al. found an increase in nuclear Nrf2 in rat myocardium after regular exercise[63]. None of these studies used skeletal muscle tissue,

making it hard to understand the changes that occur local to the muscles, which increase metabolic activity the most during exercise. This study is the first study to examine changes in Nrf2 nuclear-located protein in response to chronic exercise in skeletal muscle, particularly of different muscle groups.

Taken together, the data on KEAP1/Nrf2 currently suggests that in response to regular exercise training, Nrf2 translocates to the nucleus more at rest, but with little change in KEAP1 or Nrf2 levels in the cytosol. It is important to note that some of the findings, particularly those in the Soleus and Deep Quadriceps, may be subject to sensitivity issues, leading to type II errors. These may be due to a number of issues, namely: western blot sensitivity, exercise training stimulus, sample size, and inherent variability issues that are unaccounted for.

One major limitation to Nrf2 measurement remains unresolved in the literature: there are multiple bands for Nrf2. The predicted band should be ~69 kDa, whereas bands can show up ~100kDa[64] . Conflicting viewpoints lead to two main analyses; 1. Count both bands, as Nrf2 bands ~100 kDa are 'true', and 2. Ignore the 100kDa band and regard it as an artifact or non-specific band (particularly by antibody manufacturers). While neither of these methods appears to be the leading candidate and needs to be resolved, the approach for this study was to examine only the 69 kDa band; the rational used was that there are in fact artifact bands that exist, and there is no empirical data to suggest that the band at ~100 kDa is indeed Nrf2. Resolving this question during this study was not one of the aims, and therefore it did not warrant further investigation, but it is worth noting

that there is a possibility that this is indeed a Nrf2 isoform or Nrf2 bound to another molecule (such as s-maf), and data is being lost.

Antioxidant Responses

Log-transformed total-cell HO1 showed significant muscle main effects, with no significance in main effect for exercise or interaction effects. That is to say that HO1 did appear to differ between muscles, but exercise training did not significantly affect the levels of HO1, nor did the exercise response vary between different muscle groups. While it is well known that HO1 should differ between oxidative activity of a muscle, there are studies that suggest there should be an interaction effect of exercise and muscle[65]. While it is hard to discern why this was not the case in our study, one possible explanation is the time course of the study. HO1 does appear to be transient and responsive to stress; interestingly, HO1 has been suggested to be higher at rest in untrained individuals, representing the *response* to pro-oxidation[66]. This means that no major observable difference in the exercise group could be due to the long duration after the last stress (72 hours), but between muscles, it is logical that highly oxidative tissues require more antioxidants.

In the repeated measures ANOVA for SOD1, the exercise effect trended towards significance, but failed to reach statistical significance, with an F value of 3.887 (3 degrees of freedom), and a P-value of 0.062. This is likely due to reduced power from the study, and would likely show significance with less variability, higher exercise effect due to training, or a larger sample size. Many studies show an increase in SOD1 content and activity, particularly in oxidative muscle fibers[10,40,67,68]. Additionally, SOD1 showed a

significant muscle effect, which was expected, as SOD1 is often observed at different levels and activities based on muscle fiber type, where more oxidative-type muscles have higher levels of SOD1[67]. While not-statistically significant at alpha = 0.05, the interaction effect approached significance at a p value less than 0.1 (F = 2.215), suggesting future studies increasing power or changing the methodology may find an interaction effect with muscle and exercise.

While there is some information (of which there is contentious findings) about the exercise-induced *change in levels* of antioxidant proteins, most studies suggest that higher aerobic fibers have higher levels of antioxidants (including SOD1 and HO1) and also increase *enzyme activity* and possibly increased protein in response to exercise training[1]. Both proteins did not show an interaction effect, which would have suggested muscles respond differently to exercise in producing either protein. However, the significant muscle effects support the literature that suggests muscles differ in antioxidant levels but respond similarly to exercise. Of the two proteins, SOD1 is much closer to reaching statistical significance in an interaction effect, which may become significant when sample size is increased in future studies. Should this be the case, this contributes to some of the conflicting literature on whether the Cu,Zn-SOD isoform (SOD1) changes with exercise [citation]. Unfortunately, our findings only further the uncertainty, but provide context for future investigation.

It is important to note that SOD1 and HO1 protein levels are not the sole determinants of antioxidant capacity in cells. Rather, emphasis in the past has been placed on *activity* of the enzyme, since activity can increase despite little to no change in

protein levels. Additionally, there are far more enzymes associated with redox balance within a cell. However, the decision to measure SOD1 and HO1 protein levels was more about identifying proteins that are target proteins of Nrf2 via ARE sequences on DNA and directly related to the negative feedback loop of oxidative stress.

Nrf2/KEAP1 Ratio

One of the aims of this study was to examine whether the Nrf2/KEAP1 ratio was a significant correlator with antioxidant production, since Nrf2 is an 'activator' of antioxidant production and KEAP1 inhibits Nrf2's availability. Two ratios were used for analysis: total Nrf2/KEAP1 ratio ((nuclear Nrf2 + cytosolic Nrf2) / KEAP1) and cytosolic Nrf2/KEAP1 ratio (cytosolic Nrf2 / KEAP1). In theory, the higher the 'activator' relative to the 'inactivator' could potentially lead to increases in antioxidant levels. While this is not the best metric, since neither the ratio nor protein levels indicate actual activation of transcription, the attempt was to find an indicator that explains more of the variability that is observed in the current literature.

Using repeated-measures multivariate ANOVAs, differences between exercise groups and muscles in mice were assessed first. Results from both tests illustrated that the ratios of Nrf2/KEAP1 did not differ between exercise and sedentary groups, nor between muscles (or any interaction). While this may be hard to explain given the lack of evidence given this study design, the results suggest that the lack of significant changes in KEAP1 and Nrf2 ratio. However, it is possible that the non-significant trend of increased Nrf2 and KEAP1 meant the ratio stayed *roughly* similar. Further investigations should quantify the proteins in such a manner to more sensitively detect quantity of protein.

The current findings are relatively similar to findings from Alves in 2020, where they found exercise alone was not enough to change the Nrf2/KEAP1 ratio but did restore the ratio when combined with a fructose diet that they demonstrated decreased the Nrf2/KEAP1 ratio[69]. This suggests that perhaps at an unaltered state, exercise will not change the ratio of the two proteins' production but may help when one protein or another has a dysfunctional production. Given that these mice were apparently healthy, future studies should focus on the relationship of exercise on *dysfunctional* ratios rather than *apparently healthy* ratios.

Additionally, one of the aims of this study was to compare the Nrf2/KEAP1 ratio to levels of antioxidants in the cells to see if there were correlations in these values. Running Pearson correlations revealed that neither of the Nrf2/KEAP1 ratio metrics inclusive of both groups were correlated with HO1. Separating the data by each group and running each group as separate correlations revealed that both types of ratios still did not significantly correlate with outcome antioxidants. Examining the plots of these correlations shows that Nrf2/KEAP1 ratio is not an adequate corollary to describe changes that occur downstream in production of antioxidants. Many studies to date have shown strange behaviors in Nrf2 levels and downstream antioxidants, the proposed metric was a theoretical way to describe more aspects of the signaling pathway, since KEAP1 renders Nrf2 biologically inactive. Given the lack of significant relationships between the ratios and antioxidants, it may be presumed that other regulatory factors (DNA binding, epigenetics, post-translational modifications, etc.) may play a larger role in regulating these downstream outcomes which are not being addressed in this study.

Nonetheless, it is important to note that the relative amounts of KEAP1 to Nrf2 do not appear to correlate with the level of antioxidant production in muscle tissues.

Nuclear Nrf2 appeared to be the best correlator with antioxidant production, which makes sense given that Nrf2 must be in the nucleus to play out its role as a transcription factor and increase mRNA of target ARE genes. Our study is in line with other studies that show that nuclear Nrf2 is only mildly correlated with antioxidant production [citation]. As such, it would be beneficial to look at epigenetic-level regulators in this pathway to see whether or not that may better explain some of the discrepancies.

Limitations and Error

Many of the limitations specific to the individual proteins were mentioned previously in the discussion, but other limitations remain.

In this study, the exercise session was used from a protocol designed to illicit an oxidative stress response in each session[57]. While physical encouragement was given to mice, observational data suggested some mice were more consistent during the exercise session than others. The 'compliance' of each mice varied between mice but was not recorded in any fashion for validation. Therefore, some of the mice in the study may have smaller overall effect of exercise than others in the group, contributing to variability in the exercise group. A measure of aerobic adaptations, such as Cytochrome Oxidase activity[70], should be used in the future as a covariate to determine whether or not training *adaptation* influences the results.

Additionally, without measures of oxidative stress markers or any indication of ROS, it is hard to attribute these changes specifically to OS, rather than non-canonical activation or other unknown influences that also change with exercise training. It is known that intensity and duration affect OS, and any non-compliance from mice or error in treadmill speed may change the overall dose of OS each mouse received. Limited tissue supply (6mg – 40mg, depending on muscle) made it challenging, if not impossible, to have enough tissue to measure too many variables, particularly when measures are sensitive to preparatory methods, such as OS markers. Leaving OS marker measurements out was driven by previous findings using similar protocols demonstrating OS in this protocol, the fact that measurements were ~72 hours post-exercise (representing *basal* levels), and the priority of tissue sample allocation for proteins.

While multiplexed western blotting saved tissue, time, and money, it did have some trade-offs. First, fluorescently-labelled primary antibodies meant that there is no "amplification" of signals that typically occurs with a secondary antibody. Given the high-resolution scan (100 microns), this may not be a major limitation, but it certainly could contribute to less pronounced differences between low and high concentrations. Additionally, comparisons between proteins should not necessarily be made on an absolute quantity level when western blotting, but this remains especially true when multiplexing western blots. Different fluorophores have different brightness than others; as such, a higher intensity signal on a channel does not represent "more" protein, but it should remain relative within protein comparisons. On a similar note, it is worth pointing out that these western blots were conducted on PVDF membranes that were not "low-

fluorescence" membranes; as such, these membranes did have background fluorescence, particularly in the lower wave-length regions. While we do not suspect it affected the magnitude of response, it has the possibility to decrease overall sensitivity, despite our approach accounting for this in the analysis step. Future studies are recommended to use "low-fluorescence" PVDF membranes to make data analysis easier, quicker, and potentially more reliable.

In addition to brightness and autofluorescence, fluorophore emission spectra overlap with the filters may contribute to some signal being lost. For example, a fluorophore such as Phycoerythrin (PE) should fluoresce when excited by a 532nm laser, which should show up on the Cy3 channel (560-580nm); however, it is possible for the 635nm laser to slightly fluoresce PE, creating a band signal that appears on the Cy5 channel (655-685nm). While this remains systematic and can be considered an artifact, some excitation can lead to signals when bands are overlapping in molecular weight, which may lead to higher values. While the western blots were designed to minimize this occurrence (Appendix 3), it is worth noting that KEAP1 and Nrf2 both have bands ~69 kDa and could be susceptible to this.

Recommendations

The field of Nrf2 signal transduction is relatively new, but of high interest because of its highly conserved and broadly expressed nature, its relevance to many diseases, and potential therapeutic in exercise and pharmaceuticals. However, the current literature on exercise remains limited. Our study was the first to include KEAP1 measurements to skeletal muscle while looking at different expression patterns in

different muscles. These findings should be considered when assessing methodology approaches for future studies, as the muscle effect in antioxidant measurements may cause erroneous findings or correlations in isolation, so muscle selection is important.

Additionally, future studies should address the acute-effects of exercise on KEAP1. Many studies exist examining the pathway activation post-exercise, but do not examine short-term changes in KEAP1. This may be important to understanding the regulatory dynamics imposed on Nrf2 by KEAP1. While we don't have any reason to believe, at this point, that KEAP1 changes acutely, the non-canonical model, where p62 displaces Nrf2 as 'activation' while p62 signals KEAP1 for selective autophagy, suggests that KEAP1 could be degraded acutely in response to a buildup of p62 and/or phosphorylation of p62 by mTOR. To our knowledge, this non-canonical pathway has not been studied acutely, and requires future investigation.

Lastly, future studies should examine the modulation of intensity, duration, frequency, and modality of exercise to see whether or not these have large effects on Nrf2 signaling and subsequent antioxidant production. Oxidative stress has been shown to respond to all of these factors, and it stands to reason that Nrf2 response would change as a result to protect against different levels/types of future OS. Additionally, more studies are needed to address the time-course difference.

REFERENCES

1. Power SK, Jackson MJ. Exercise-Induced Oxidative Stress: Cellular Mechanisms and Impact on Muscle Force Production. Physiological reviews. 2008;88(4):1243–1276. doi:10.1152/physrev.00031.2007

2. Remacle J, Raes M, Toussaint O, Renard P, Rao G. Low levels of reactive oxygen species as modulators of cell function. Mutation Research/DNAging. 1995;316(3):103–122. doi:10.1016/0921-8734(95)90004-7

3. Halliwell B. Reactive oxygen species in living systems: Source, biochemistry, and role in human disease. The American Journal of Medicine. 1991;91(3, Supplement 3):S14–S22. (Oxidants And Antioxidants: Pathophysiologic Determinants and Therapeutic Agents). doi:10.1016/0002-9343(91)90279-7

4. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. Antioxidants & Redox Signaling. 2014;20(7):1126–1167. doi:10.1089/ars.2012.5149

5. Yang H-C, Cheng M-L, Ho H-Y, Tsun-Yee Chiu D. The microbicidal and cytoregulatory roles of NADPH oxidases. Microbes and Infection. 2011;13(2):109–120. doi:10.1016/j.micinf.2010.10.008

6. Matlung HL, Bakker ENTP, VanBavel E. Shear Stress, Reactive Oxygen Species, and Arterial Structure and Function. Antioxidants & Redox Signaling. 2009;11(7):1699–1709. doi:10.1089/ars.2008.2408

7. Kerksick C, Willoughby D. The Antioxidant Role of Glutathione and N-Acetyl-Cysteine Supplements and Exercise-Induced Oxidative Stress. Journal of the International Society of Sports Nutrition. 2005;2:38. doi:10.1186/1550-2783-2-2-38

8. Mason SA, Morrison D, McConell GK, Wadley GD. Muscle redox signalling pathways in exercise. Role of antioxidants. Free Radical Biology and Medicine. 2016;98:29–45. (Human Performance and Redox Signaling in Health and Disease). doi:10.1016/j.freeradbiomed.2016.02.022

9. Fisher G, Schwartz DD, Quindry J, Barberio MD, Foster EB, Jones KW, Pascoe DD. Lymphocyte enzymatic antioxidant responses to oxidative stress following high-intensity interval exercise. Journal of Applied Physiology (Bethesda, Md.: 1985). 2011;110(3):730–737. doi:10.1152/japplphysiol.00575.2010

10. Powers SK, Criswell D, Lawler J, Ji LL, Martin D, Herb RA, Dudley G. Influence of exercise and fiber type on antioxidant enzyme activity in rat skeletal muscle. The American Journal of Physiology. 1994;266(2 Pt 2):R375-380.

11. Powers SK, Ji LL, Leeuwenburgh C. Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. Medicine & Science in Sports & Exercise. 1999;31(7):987–997.

12. Zhang DD, Hannink M. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Molecular and Cellular Biology. 2003;23(22):8137–8151. doi:10.1128/mcb.23.22.8137-8151.2003

13. Done AJ, Traustadóttir T. Nrf2 mediates redox adaptations to exercise. Redox Biology. 2016;10:191–199. doi:10.1016/j.redox.2016.10.003

14. García-Mesa Y, López-Ramos JC, Giménez-Llort L, Revilla S, Guerra R, Gruart A, LaFerla FM, Cristòfol R, Delgado-García JM, Sanfeliu C. Physical Exercise Protects Against Alzheimer's Disease in 3xTg-AD Mice. Journal of Alzheimer's Disease. 2011;24(3):421–454. doi:10.3233/JAD-2011-101635

15. Shanmugam G, Challa AK, Devarajan A, Athmanathan B, Litovsky SH, Krishnamurthy P, Davidson CJ, Rajasekaran NS. Exercise Mediated Nrf2 Signaling Protects the Myocardium From Isoproterenol-Induced Pathological Remodeling. Frontiers in Cardiovascular Medicine. 2019;6:68. doi:10.3389/fcvm.2019.00068

16. Krajka-Kuźniak V, Paluszczak J, Baer-Dubowska W. The Nrf2-ARE signaling pathway: An update on its regulation and possible role in cancer prevention and treatment. Pharmacological Reports. 2017;69(3):393–402. doi:10.1016/j.pharep.2016.12.011

17. Gounder SS, Kannan S, Devadoss D, Miller CJ, Whitehead KS, Odelberg SJ, Firpo MA, Iii RP, Hoidal JR, Abel ED, et al. Impaired Transcriptional Activity of Nrf2 in Age-

Related Myocardial Oxidative Stress Is Reversible by Moderate Exercise Training. PLOS ONE. 2012;7(9):e45697. doi:10.1371/journal.pone.0045697

18. Cao H, Wang L, Chen B, Zheng P, He Y, Ding Y, Deng Y, Lu X, Guo X, Zhang Y, et al. DNA Demethylation Upregulated Nrf2 Expression in Alzheimer's Disease Cellular Model. Frontiers in Aging Neuroscience. 2015;7:244. doi:10.3389/fnagi.2015.00244

19. Uruno A, Furusawa Y, Yagishita Y, Fukutomi T, Muramatsu H, Negishi T, Sugawara A, Kensler TW, Yamamoto M. The Keap1-Nrf2 System Prevents Onset of Diabetes Mellitus. Molecular and Cellular Biology. 2013;33(15):2996–3010. doi:10.1128/MCB.00225-13

20. Huang T-T, Hao D-L, Wu B-N, Mao L-L, Zhang J. Uric acid demonstrates neuroprotective effect on Parkinson's disease mice through Nrf2-ARE signaling pathway. Biochemical and Biophysical Research Communications. 2017;493(4):1443–1449. doi:10.1016/j.bbrc.2017.10.004

21. Aguiar AS, Duzzioni M, Remor AP, Tristão FSM, Matheus FC, Raisman-Vozari R, Latini A, Prediger RD. Moderate-Intensity Physical Exercise Protects Against Experimental 6-Hydroxydopamine-Induced Hemiparkinsonism Through Nrf2-Antioxidant Response Element Pathway. Neurochemical Research. 2016;41(1–2):64–72. doi:10.1007/s11064-015-1709-8

22. Kim T, Yang Q. Peroxisome-proliferator-activated receptors regulate redox signaling in the cardiovascular system. World Journal of Cardiology. 2013;5(6):164–174. doi:10.4330/wjc.v5.i6.164

23. Jiang H, Miao Y, Wang Y, Zhao M, Feng Z, Yu X, Liu J, Zang W. Aerobic interval training protects against myocardial infarction-induced oxidative injury by enhancing antioxidase system and mitochondrial biosynthesis. Clinical & Experimental Pharmacology & Physiology. 2014;41(3):192–201. doi:10.1111/1440-1681.12211

24. Maritim AC, Sanders RA, Watkins JB. Diabetes, oxidative stress, and antioxidants: A review. Journal of Biochemical and Molecular Toxicology. 2003;17(1):24–38. doi:10.1002/jbt.10058

25. Suzuki M, Otsuki A, Keleku-Lukwete N, Yamamoto M. Overview of redox regulation by Keap1–Nrf2 system in toxicology and cancer. Current Opinion in Toxicology. 2016;1:29–36. (Oxidative Toxicology). doi:10.1016/j.cotox.2016.10.001

26. Tong Y-H, Zhang B, Fan Y, Lin N-M. Keap1–Nrf2 pathway: A promising target towards lung cancer prevention and therapeutics. Chronic Diseases and Translational Medicine. 2015;1:175–186. doi:10.1016/j.cdtm.2015.09.002

27. Yu S, Khor TO, Cheung K-L, Li W, Wu T-Y, Huang Y, Foster BA, Kan YW, Kong A-N. Nrf2 Expression Is Regulated by Epigenetic Mechanisms in Prostate Cancer of TRAMP Mice. PLOS ONE. 2010;5(1):e8579. doi:10.1371/journal.pone.0008579

28. Turrens JF. Superoxide Production by the Mitochondrial Respiratory Chain. Bioscience Reports. 1997;17(1):3–8. doi:10.1023/A:1027374931887

29. La Favor Justin D., Dubis Gabriel S., Yan Huimin, White Joseph D., Nelson Margaret A.M., Anderson Ethan J., Hickner Robert C. Microvascular Endothelial Dysfunction in Sedentary, Obese Humans Is Mediated by NADPH Oxidase. Arteriosclerosis, Thrombosis, and Vascular Biology. 2016;36(12):2412–2420. doi:10.1161/ATVBAHA.116.308339

30. Davies MJ. Singlet oxygen-mediated damage to proteins and its consequences. Biochemical and Biophysical Research Communications. 2003;305(3):761–770. doi:10.1016/S0006-291X(03)00817-9

31. Mueller S, Riedel H-D, Stremmel W. Determination of Catalase Activity at Physiological Hydrogen Peroxide Concentrations. Analytical Biochemistry. 1997;245(1):55–60. doi:10.1006/abio.1996.9939

32. Arnér ESJ, Holmgren A. Physiological functions of thioredoxin and thioredoxin reductase. European Journal of Biochemistry. 2000;267(20):6102–6109. doi:10.1046/j.1432-1327.2000.01701.x

33. Holmgren A. Thioredoxin. Annual Review of Biochemistry. 1985;54(1):237–271. doi:10.1146/annurev.bi.54.070185.001321

34. Fernandes AP, Holmgren A. Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. Antioxidants & Redox Signaling. 2004;6(1):63–74. doi:10.1089/152308604771978354

35. Rhee SG. Overview on Peroxiredoxin. Molecules and Cells. 2016;39(1):1–5. doi:10.14348/molcells.2016.2368

36. Meister A, Anderson ME. Glutathione. Annual Review of Biochemistry. 1983;52:711–760. doi:10.1146/annurev.bi.52.070183.003431

37. Waugh WH. Inhibition of iron-catalyzed oxidations by attainable uric acid and ascorbic acid levels: therapeutic implications for Alzheimer's disease and late cognitive impairment. Gerontology. 2008;54(4):238–243. doi:10.1159/000122618

38. Sedlak TW, Saleh M, Higginson DS, Paul BD, Juluri KR, Snyder SH. Bilirubin and glutathione have complementary antioxidant and cytoprotective roles. Proceedings of the National Academy of Sciences. 2009;106(13):5171–5176. doi:10.1073/pnas.0813132106

39. Loboda A, Damulewicz M, Pyza E, Jozkowicz A, Dulak J. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cellular and Molecular Life Sciences. 2016;73(17):3221–3247. doi:10.1007/s00018-016-2223-0

40. Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, Sou Y-S, Ueno I, Sakamoto A, Tong KI, et al. The selective autophagy substrate p62 activates the

stress responsive transcription factor Nrf2 through inactivation of Keap1. Nature Cell Biology. 2010;12(3):213–223. doi:10.1038/ncb2021

41. Patwell DM, McArdle A, Morgan JE, Patridge TA, Jackson MJ. Release of reactive oxygen and nitrogen species from contracting skeletal muscle cells. Free Radical Biology and Medicine. 2004;37(7):1064–1072. doi:10.1016/j.freeradbiomed.2004.06.026

42. Sindler AL, Reyes R, Chen B, Ghosh P, Gurovich AN, Kang LS, Cardounel AJ, Delp MD, Muller-Delp JM. Age and exercise training alter signaling through reactive oxygen species in the endothelium of skeletal muscle arterioles. Journal of Applied Physiology. 2013;114(5):681–693. doi:10.1152/japplphysiol.00341.2012

43. Matoba T, Shimokawa H. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in animals and humans. Journal of Pharmacological Sciences. 2003;92(1):1–6. doi:10.1254/jphs.92.1

44. Kim Y-W, Byzova TV. Oxidative stress in angiogenesis and vascular disease. Blood. 2014;123(5):625–631. doi:10.1182/blood-2013-09-512749

45. Granger DN, Kvietys PR. Reperfusion injury and reactive oxygen species: The evolution of a concept. Redox Biology. 2015;6:524–551. doi:10.1016/j.redox.2015.08.020

46. Kanter MM, Hamlin RL, Unverferth DV, Davis HW, Merola AJ. Effect of exercise training on antioxidant enzymes and cardiotoxicity of doxorubicin. Journal of Applied Physiology. 1985;59(4):1298–1303. doi:10.1152/jappl.1985.59.4.1298

47. Morgan MJ, Liu Z. Crosstalk of reactive oxygen species and NF-κB signaling. Cell Research. 2011;21(1):103–115. doi:10.1038/cr.2010.178

48. El-Naa MM, El-Refaei MF, Nasif WA, Abduljawad SH, El-Brairy AI, El-Readi MZ. In-vivo antioxidant and anti-inflammatory activity of rosiglitazone, a peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists in animal model of bronchial asthma. The Journal of Pharmacy and Pharmacology. 2015;67(10):1421–1430. doi:10.1111/jphp.12445

49. Asghar M, George L, Lokhandwala MF. Exercise decreases oxidative stress and inflammation and restores renal dopamine D1 receptor function in old rats. American Journal of Physiology-Renal Physiology. 2007;293(3):F914–F919. doi:10.1152/ajprenal.00272.2007

50. Fardoun RZ, Asghar M, Lokhandwala M. Role of oxidative stress in defective renal dopamine D1 receptor-G protein coupling and function in old Fischer 344 rats. American Journal of Physiology-Renal Physiology. 2006;291(5):F945–F951. doi:10.1152/ajprenal.00111.2006

51. George L, Lokhandwala MF, Asghar M. Exercise activates redox-sensitive transcription factors and restores renal D1 receptor function in old rats. American Journal of Physiology-Renal Physiology. 2009;297(5):F1174–F1180. doi:10.1152/ajprenal.00397.2009

52. Merry T, Ristow M. Nuclear factor erythroid-derived 2-like 2 (NFE2L2, Nrf2) mediates exercise-induced mitochondrial biogenesis and the anti-oxidant response in mice - Merry - 2016 - The Journal of Physiology - Wiley Online Library. [accessed 2018 Nov 9]. https://physoc.onlinelibrary.wiley.com/doi/full/10.1113/JP271957

53. Yu Q, Xia Z, Liong E, Tipoe G. Chronic aerobic exercise improves insulin sensitivity and modulates Nrf2 and NF-κB/IκBα pathways in the skeletal muscle of rats fed with a high fat diet. Molecular Medicine Reports. 2019;20(6):4963–4972. doi:10.3892/mmr.2019.10787

54. Rahimi G, Heydari S, Rahimi B, Abedpoor N, Niktab I, Safaeinejad Z, Peymani M, Seyed Forootan F, Derakhshan Z, Esfahani MHN, et al. A combination of herbal compound (SPTC) along with exercise or metformin more efficiently alleviated diabetic complications through down-regulation of stress oxidative pathway upon activating Nrf2-Keap1 axis in AGE rich diet-induced type 2 diabetic mice. Nutrition & Metabolism. 2021 [accessed 2021 Feb 26];18. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7816367/. doi:10.1186/s12986-021-00543-6

55. Huang Y, Li W, Su Z, Kong A-NT. The complexity of the Nrf2 pathway: Beyond the antioxidant response. The Journal of nutritional biochemistry. 2015;26(12):1401–1413. doi:10.1016/j.jnutbio.2015.08.001

56. Li T, He S, Liu S, Kong Z, Wang J, Zhang Y. Effects of different exercise durations on Keap1-Nrf2-ARE pathway activation in mouse skeletal muscle. Free Radical Research. 2015;49(10):1269–1274. doi:10.3109/10715762.2015.1066784

57. Schefer V, Talan MI. Oxygen consumption in adult and aged C57BL/6J mice during acute treadmill exercise of different intensity. Experimental Gerontology. 1996;31(3):387–392. doi:10.1016/0531-5565(95)02032-2

58. Finch H. Comparison of the Performance of Nonparametric and Parametric MANOVA Test Statistics when Assumptions Are Violated. Methodology. 2005;1(1):27–38. doi:10.1027/1614-1881.1.1.27

59. Sheehan-Holt JK. MANOVA Simultaneous Test Procedures: The Power and Robustness of Restricted Multivariate Contrasts. Educational and Psychological Measurement. 1998;58(6):861–881. doi:10.1177/0013164498058006001

60. Gandoy-Fieiras N, Gonzalez-Juanatey JR, Eiras S. Myocardium Metabolism in Physiological and Pathophysiological States: Implications of Epicardial Adipose Tissue and Potential Therapeutic Targets. International Journal of Molecular Sciences. 2020;21(7):2641. doi:10.3390/ijms21072641

61. Charles JP, Cappellari O, Hutchinson JR. A Dynamic Simulation of Musculoskeletal Function in the Mouse Hindlimb During Trotting Locomotion. Frontiers in Bioengineering and Biotechnology. 2018 [accessed 2021 Feb 20];6. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5964171/. doi:10.3389/fbioe.2018.00061

62. Harms SJ, Hickson RC. Skeletal muscle mitochondria and myoglobin, endurance, and intensity of training. Journal of Applied Physiology: Respiratory, Environmental and Exercise Physiology. 1983;54(3):798–802. doi:10.1152/jappl.1983.54.3.798

63. Sun M, Huang C, Wang C, Zheng J, Zhang P, Xu Y, Chen H, Shen W. Ginsenoside Rg3 improves cardiac mitochondrial population quality: Mimetic exercise training. Biochemical and Biophysical Research Communications. 2013;441(1):169–174. doi:10.1016/j.bbrc.2013.10.039

64. Lau A, Tian W, Whitman SA, Zhang DD. The Predicted Molecular Weight of Nrf2: It Is What It Is Not. Antioxidants & Redox Signaling. 2013;18(1):91–93. doi:10.1089/ars.2012.4754

65. Vesely MJJ, Sanders R, Green CJ, Motterlini R. Fibre type specificity of haem oxygenase-1 induction in rat skeletal muscle. FEBS Letters. 1999;458(2):257–260. doi:https://doi.org/10.1016/S0014-5793(99)01129-1

66. Niess AM, Passek F, Lorenz I, Schneider EM, Dickhuth H-H, Northoff H, Fehrenbach E. Expression of the antioxidant stress protein heme oxygenase-1 (HO-1) in human leukocytes: Acute and adaptational responses to endurance exercise. Free Radical Biology and Medicine. 1999;26(1):184–192. doi:10.1016/S0891-5849(98)00192-0

67. Higuchi M, Cartier L, Chen M, Holloszy JO. Superoxide Dismutase and Catalase in Skeletal Muscle: Adaptive Response to Exercise1. Journal of Gerontology. 1985;40(3):281–286. doi:10.1093/geronj/40.3.281

68. Leeuwenburgh C, Fiebig R, Chandwaney R, Ji LL. Aging and exercise training in skeletal muscle: responses of glutathione and antioxidant enzyme systems. The American Journal of Physiology. 1994;267(2 Pt 2):R439-445. doi:10.1152/ajpregu.1994.267.2.R439

69. Alves R, Suehiro CL, Oliveira FG de, Frantz EDC, Medeiros RF de, Vieira R de P, Martins M de A, Lin CJ, Nobrega ACL da, Toledo-Arruda AC de. Aerobic exercise modulates cardiac NAD(P)H oxidase and the NRF2/KEAP1 pathway in a mouse model of chronic fructose consumption. Journal of Applied Physiology (Bethesda, Md.: 1985). 2020;128(1):59–69. doi:10.1152/japplphysiol.00201.2019

70. Henriksson J, Reitman JS. Time Course of Changes in Human Skeletal Muscle Succinate Dehydrogenase and Cytochrome Oxidase Activities and Maximal Oxygen Uptake with Physical Activity and Inactivity. Acta Physiologica Scandinavica. 1977;99(1):91–97. doi:https://doi.org/10.1111/j.1748-1716.1977.tb10356.x

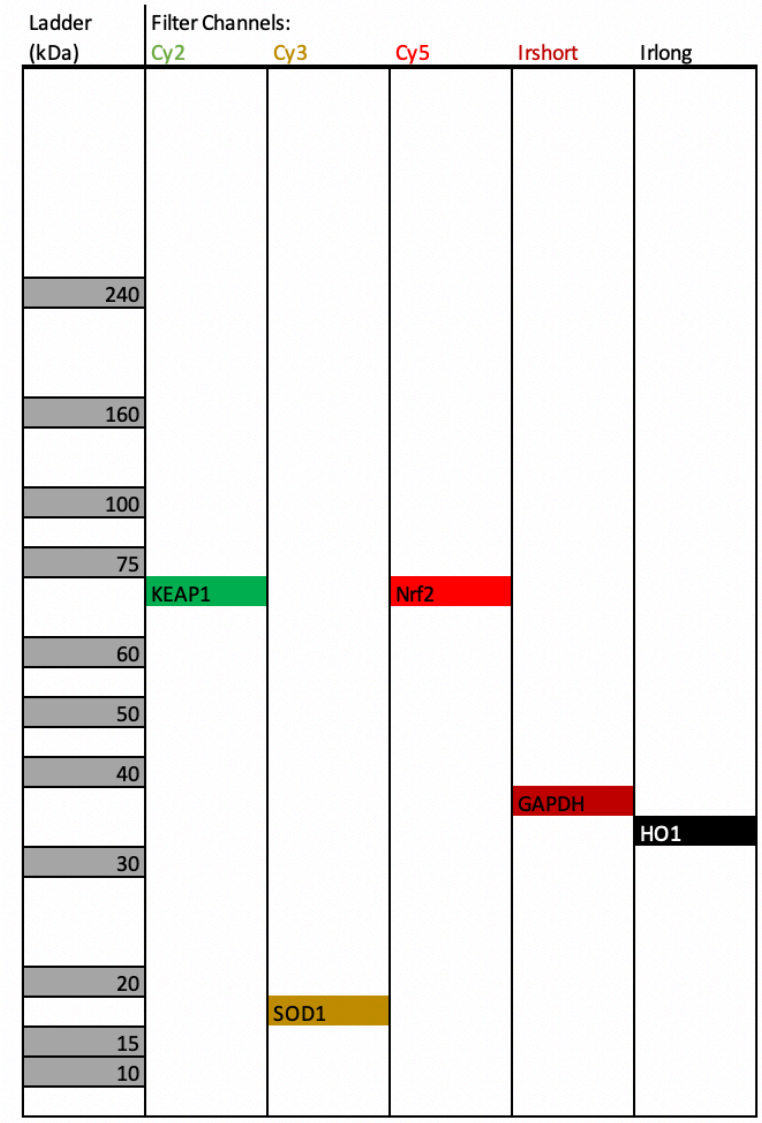
APPENDIX A. TREADMILL PROTOCOL FOR MICE EXERCISE TRAINING

Week	Incline (degrees)	Speed (m/min)	Time (min)
1	0	15	45
2	0	15	45
3	0	15	60
4	0	15	60
5	2	15	60
6	2	15	60
7*	4	15	60
8*	4	15	60

Acclimation was be done the week prior to week 1, consisting of three low speed running sessions of 20 minutes, increasing speed each session up to 15 m/min for the final session.

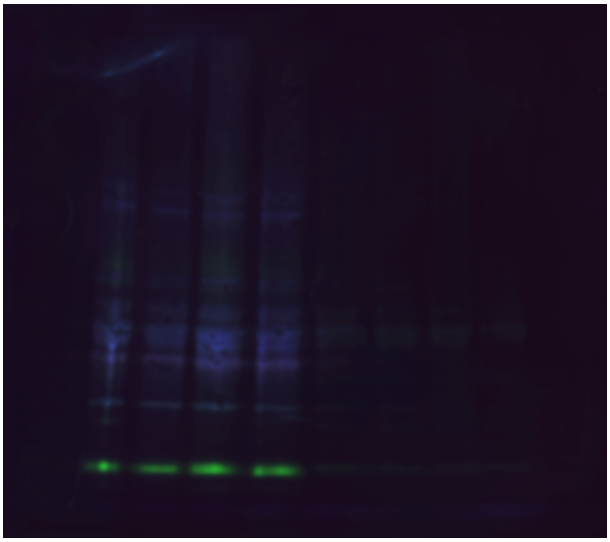
*Weeks 7 and 8 are designed to be roughly 65% VO_2max for the C57/BL6 mouse strain.

APPENDIX B. ARRANGEMENT OF FLUOROPHORES AND FILTERS FOR MULTIPLEXED WESTERN BLOTS

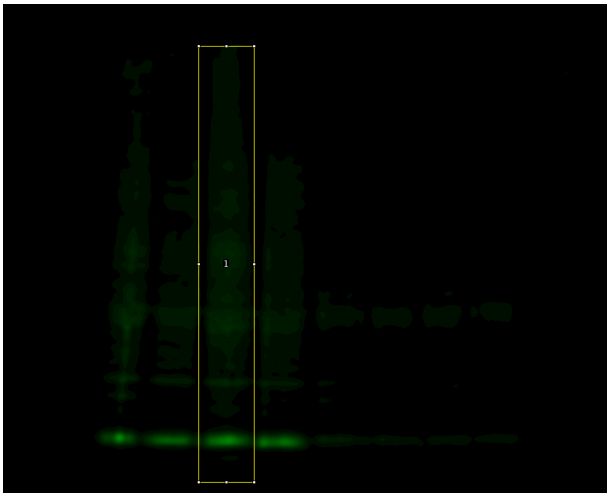


Lasers	Channels	Wavelengths (nm)	Fluorophore
488	Cy2LP	515-535	KEAP1 Alexa Fluor 488
532	Cy3LP	560-580	SOD1 Phycoerythrin
635	Cy5LP	655-685	Nrf2 Alexa Fluor 647
685	IR Short	710-730	GAPDH Alexa Fluor 680
785	IR Long	810-840	HO1 Alexa Fluor 790

APPENDIX C. WESTERN BLOT IMAGE PROCESSING

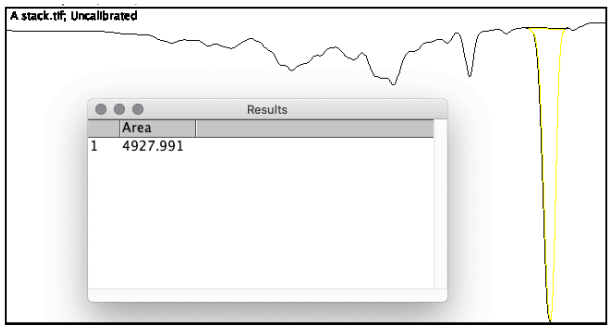


The Amersham Typhoon imager scanned one channel at a time, with its appropriate laser combination (Appendix 2). Each scan was a density measurement that was on grayscale. Image J was used to convert grayscale images to 5 separate color channels, which were overlapped to represent the composite picture. Each color represents an individual channel (in this case, protein)



Each channel was isolated to look at density measurements without interference from other channels. Rectangles were used to outline each lane, which was the set range for individual density measurements.

It is important to note that each channel was 'read' as the original file, where the colors on the display *do not* change the underlying data.



Each lane received its own absorption measurement chart (X asix: left to right is a single lane, top to bottom; Y axis: absorption). Each individual chart had prominent 'dips' corresponding with protein bands, of which the area was used as the raw measurement for that band.